SW01.S1 Organization of Eukaryotic Genomes (I-S1)

SW01.S1–1
Polycomb proteins and the regulation of nuclear organization during development
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Polycomb Group (PcG) and trithorax group (trxG) proteins form multimeric protein complexes that regulate chromatin via histone modifications and modulation of nucleosome remodeling activities, targeted to specific cis-regulatory elements named PcG response elements (PREs). These proteins maintain the memory of regulatory states of crucial developmental target genes, such as Hox genes. They can also dynamically bind to other genes and affect cell proliferation and differentiation in many physiological processes and in disease.

Furthermore, PcG proteins play a role in nuclear organization. Staining for these proteins shows a distribution in foci called Polycomb bodies. Polycomb bodies are the physical sites of Polycomb-mediated silencing. Moreover, endogenous PcG target loci can frequently colocalize in the cell nucleus. These contacts depend on Polycomb proteins and stabilize epigenetic gene silencing. Thus, the three-dimensional organization of the genome contributes to epigenetic memory of gene expression states. Here, I will discuss developmental dynamics of PcG protein function in flies and their role in regulating chromosome architecture.

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References

SW01.S1–2
Organizing the genome through development: anchoring of heterochromatin to the nuclear envelope through histone H3K9 methylation
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Heterochromatin comes in several forms and becomes the dominant form of chromatin as cells terminally differentiate. At least one class of heterochromatin is positioned adjacent to the nuclear lamina. We have created a system in which we can track gene position in developing C. elegans by live fluorescence microscopy. We have found that in differentiated cells, developmentally regulated promoters are at the nuclear periphery when repressed, and shift inwards when active. In early embryonic cells gene positions are not fixed. Using an in vivo model of fluorescently tagged heterochromatin, we have screened for factors that are necessary for anchoring heterochromatin to the nuclear lamina. We find that its peripheral anchoring is a direct consequence of sequential methylation reactions by two enzymes that modify histone H3 lysine 9. Mono- and di-methylation of H3K9 mediates anchoring, while silencing of the array requires H3K9 trimethylation. A further screen has identified a novel chromodomain protein that mediates anchorage by binding H3K9me, to link chromatin bearing this mark to the nuclear envelope. The physiological effects of disrupting the spatial organization of chromatin through loss of the anchoring machinery will be discussed.

SW01.S1–3
Gene regulation in the 3D genome
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Pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) were recently shown to organize their chromosomes into topological domains that are largely invariant between cell types. Here, we applied 4C technology and combined ChIP-seq with Hi-C data to uncover unique higher-order topological features of the PSC genome. In addition, we will present a new strategy to map the parts of the genome that are associated with pericentromeric heterochromatin. We will discuss what these regions are and how they vary between different tissues.

SW01.S1–4
Elusive active chromatin hubs: nuclear compartments, folded chromatin domains or rigid complexes of regulatory elements?
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Development of a panel of the so-called C-methods – experimental protocols that allow the study of the 3D organization of the eukaryotic genome – has permitted to make observations resulting in a new concept in molecular genetics. In became evident that the 3D genome organization constitutes a part of epigenetic mechanisms essential for maintaining the identity of differentiated cells. In this respect, the assembly of distant regulatory elements in common activating complexes – active chromatin hubs – appears to be of primary importance. Here we show that one of the principal assumptions behind the C-methods is not correct. All C-methods are based on the “proximity ligation” which is
preferential cross-ligation of interacting DNA fragments that remain joined by protein bridges after solubilization from formaldehyde-fixed nuclei. We show that the proximity ligation in the 3C procedure really occurs within non-lysed nuclei inside a cage formed by cross-linked chromatin fibers. This finding allows a new interpretation of the results of 3C analysis. Our data suggest that regulatory elements participating in formation of an active chromatin hub do not necessarily form a common complex stabilized by protein bridges, but rather are recruited to the same nuclear compartment where they retain a certain degree of mobility. This model is further supported by demonstration that treatment of nuclei by agents inducing decompaction of chromatin performed before the fixation with formaldehyde results in a decrease of 3C signal.

**SW01.S1–5**

The roles of cohesin and CTCF for shaping the chromatin fiber

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Recent studies of genome-wide chromatin interactions have revealed that the human genome is partitioned into many self-associating topological domains but how these structures are formed is not understood.

Candidate factors that are very abundant all over the genome are CTCF and cohesin. The chromatin insulator CTCF is known to play a role in chromosomal long-range contacts for some time. We have shown that the cohesin complex, well known to establish sister chromatid cohesion by tethering DNA strands together, can also establish chromosomal contacts in *cis*. Cohesin and CTCF largely colocalize throughout the genome and are thought to interact functionally to promote chromatin insulation and chromosomal interactions.

To determine the respective roles of cohesin and CTCF in the higher order chromatin architecture of human cells, we removed the cohesin complex from interphase chromatin by proteolytic cleavage and examined changes in local chromosomal organization using 3C-sequencing and genome-wide using Hi-C. We observed a general loss of local chromosomal interactions upon disruption of the cohesin complex, but the topological domains remain largely intact. However, we found that depletion of CTCF by RNA interference not only reduced intra-domain interactions but also increased inter-domain interactions. Furthermore, distinct groups of genes become mis-regulated upon depletion of cohesin or CTCF. While CTCF might influence the promoters of genes directly, cohesin depletion likely affects genes by altered chromosomal long-range interactions. Taken together, these observations suggest that CTCF and cohesin contribute in different ways to chromatin organization and gene regulation.

**SW01.S1–6**

DNA replication origins: genetic and epigenetic features, and relationships with cell identity

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In multicellular eukaryotes, 30 000–50 000 DNA replication origins (Oris) are activated at each cell division to allow accurate genome duplication. Until recently, only a very few of them were identified. We used both genome-wide and single DNA molecule approaches to obtain insights into the genetic and epigenetic features of origins, their chromosomal organization and usage during the cell cycle. Mouse cells, Drosophila cells and *C. elegans* embryos were analyzed. Following purification of RNA-primed nascent DNA strands, mapping of replication origins was performed by Q-PCR analysis, tiling arrays and high-throughput sequencing.

We identified consensus elements called OGRE (Origin G-rich Repeated Elements) that are present in 67–90% of the DNA replication origins from Drosophila to human cells, respectively. OGREs form G-quadruplex structures preferentially at Oris and might be structural elements controlling the choice or activation of replication origins. OGRE density along chromosomes correlated with previously published replication timing data. Our analyses show that initiation of DNA synthesis takes place precisely at 160 bp (Drosophila) and 280 bp (mouse) downstream from the OGRE. We also found that in most CpG islands an OGRE is positioned in opposite orientation on each of the two DNA strands, and detected two sites of initiation of DNA synthesis upstream or downstream of each OGRE. Conversely, Oris not associated with CpG islands have a single initiation site. Our findings support the hypothesis that specific sequences, associated with structural elements, are involved in origin recognition and function in metazoans, driving the replication start site downstream from these. Their association with other chromosomal features will be also discussed.

Potential replication origins are site-specific but their usage is flexible. Chromosomes are organized in spaced 50 kbp replicons containing 4–5 potential replication origins where a single one is activated in a flexible manner at each cell cycle, with a mean inter-origin spacing of 130 Kbp. This organization provides flexibility of the replication program during the cell cycle, or according to cell differentiation. Accordingly, the usage of DNA replication origins of mouse nuclei can be entirely reprogrammed in embryonic extracts.

**SW01.S1–7**

Modelling large-scale organization of chromatin: a tale of the HoxB locus organization in mouse ES cells

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Long-range chromatin interactions have been identified as major regulators of gene expression and chromosome organization. To investigate the mechanisms of chromatin folding and interaction, we used as model system a 1 Mb genomic region containing active genes and a cluster of HoxB genes, which are repressed by Polycomb-repressor complexes in embryonic stem (ES) cells.

We used a simple polymer model, the Strings & Binders Switch (SBS) model (Nicodemi et al. 2008, Nicodemi and Prisco, 2009, Barbieri et al. 2012), to determine the different scenarios of 3D folding of the HoxB genomic region, in the presence of active transcription factories and Polycomb bodies, separately or together. Active genes are known to interact at active transcription factories, whereas Polycomb-repressed genes associate with poised transcription factories or Polycomb bodies (Ferrai et al. 2010, Brookes et al. 2012).

Using epigenetic mapping, we classified all the genes across the HoxB locus (1 Mb genomic region), according to their activity status in ES cells (active, Polycomb-repressed or inactive).
Using high-resolution imaging by cryo-FISH, we find agreement with one of the scenarios predicted by the model, in which both active and poised transcription factories contribute to the 3D folding of the locus. We also identify novel examples of gene co-association at the same transcription factory, and explore the effects of genomic context on the 3D interaction behavior of single genes.

References

Large scale chromatin organization: the case of PcG bodies
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The interphase nucleus of eukaryotes is a highly compartmentalized organelle. Its inner content can be categorized into a chromatin compartment and an interchromatin compartment. The interchromatin compartment harbors numerous distinct protein/RNA-based subcompartments that contain little or no DNA. Such nuclear subcompartments offer a mechanism by which to concentrate and spatially segregate nuclear activities. For example, nuclear bodies represent the local accumulations of a set of resident proteins and RNAs in the interchromatin compartment and offer a microenvironment that can improve the efficiency and fidelity of protein-DNA or protein-RNA interactions that are important for gene expression. Specifically, a Polycomb (PcG) body is considered to be the accumulation of PcG proteins, and it has been proposed that the PcG body acts as a gene silencing factory in the interchromatin compartment. However, the structural basis of the PcG body is still under debate. Diverging reports on protein-based versus chromatin-based nature of the PcG body were published. In order to expand the information on the nature of the PcG bodies in U-2 OS cells, we focused on the behaviour of the PcG proteins encompassed in fluorescent PcG bodies under conditions of hyperosmotically induced molecular crowding that allows to differentiate between interchromatin and chromatin compartments. We observed under these conditions that the PcG bodies disappeared, but persisted as nuclear domains characterized by accumulations of DNA. We found that the disappearance of PcG proteins from the original PcG bodies was associated with their hyper-phosphorylation. Importantly, fluorescence microscopy results showed that the changes observed in hyperosmotically treated cells were quickly reversible after reincubation of cells in normal medium. We conclude that the PcG body in U-2 OS cells is not a nuclear body, but a chromatin domain.

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Drosophila ELYS protein affects chromosome architecture in interphase nucleus
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Nuclear pore complexes (NPCs) are composed of approximately 30 nucleoporins forming the gates for nucleocytoplasmic transport. Recent data in yeast, mammals and Drosophila indicate that NPCs contact chromatin and thus might influence chromosome architecture (for review see Liang and Hetzer, 2011). One of the nucleoporins, ELYS, participates in the NPC assembly at the end of mitosis targeting Nup107-Nup160 subcomplex to nuclear membranes and attaching AT-rich chromatin (Rasala et al, 2008). ELYS orthologs is known from nematode to human.

ELYS ortholog in Drosophila encoded by CG14215 gene was not characterized previously. We generated rabbit polyclonal antibodies against CG14215 C-terminal end and have shown that CG14215 protein (dELYS) is located mostly at the nuclear envelope in S2 cells. Moreover, RNAi deplelement of lamin DmO leads to NPCs as well as to dELYS clustering at the nuclear envelope with dELYS and NPCs colocalization, while dELYS depletion results in the weakening of anti-NPC (Mab414) antibodies staining. These data indicate that dELYS is a component of nuclear pore complex in Drosophila as well as in other organisms.

Fluorescence in situ hybridization using approximately 35-kb probe for silent genome region in dELYS RNAi-treated S2 cells results in substantial shift in locus position from nuclear periphery to nuclear interior. This shift was comparable with that seen after lamin DmO RNAi. These data indicate that dELYS might participate in determination of chromosome architecture in the interphase nucleus.

RNA remodeling and the biogenesis of the dosage compensation complex in Drosophila
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The male-specific-lethal dosage compensation complex (MSL-DCC) selectively associates with the X chromosome in male Drosophila flies, where it activates the transcription in the two-fold range. Accordingly, the mRNA synthesized form the single X in males to matches the combined output of the two female X chromosomes.

The MSL-DCC) consists of five so-called male-specific lethal (MSL) proteins and two long, non-coding roX RNAs, whose function is enigmatic. It is assumed that the RNA helicase MLE, itself an MSL subunit, is involved in the assembly of the ribonucleoprotein regulator. The genome-wide chromosomal association profiles for the MLE shows MLE highly enriched at HAS closely co-localizing with the specific DCC subunit, MSL2. We therefore hypothesized a role of MLE for the targeting and/or assembly of the DCC at HAS.

Our biochemical analyses support for this idea. We determined the secondary structure of roX2 and mapped specific interaction sites for MLE in vitro. Our current study suggests that the assembly of the ribonucleoprotein MSL-DCC is initiated by an
obligatory, energy-consuming RNA remodeling step, during which MLE recognizes the SLroX structure and converts it into a more extended form. Accordingly, the stable, low energy SL conformation roX2 adopts in vitro represents an inactive, closed conformation, which needs to be actively opened to expose binding sites for MSL proteins. We found that the interaction of MSL2 with roX RNA is promoted by the ATP-dependent action of MLE. MSL2 provides a connection to an MSL1/MOF/MSL3 module via its association with MSL1. The MLE-dependent association of MSL2 with a functionally relevant structure on roX RNA may, therefore, be a key step in the biogenesis of the MSL-DCC.

**SW01.S1–11**
Transcription contributes to efficient chromosome segregation in *Streptococcus pneumoniae*

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The forces that drive the essential process of chromosome segregation are particularly poorly understood in spherical and oval-shaped bacteria. We have previously shown that the DNA-binding protein ParB and the condensin SMC (structural maintenance of chromosomes) promote efficient chromosome segregation in the oval-shaped human pathogen *Streptococcus pneumoniae*. However, viability of most cells is unaffected in parB and smc mutants indicating the presence of additional mechanisms controlling chromosome segregation. Using time-lapse microscopy together with total internal reflection fluorescence microscopy we have characterized the dynamics of chromosome segregation during the pneumococcal cell cycle. While origin regions segregated relatively rapidly, the general bulk of the nucleoids increased and separated more gradually during growth. These observations suggest that chromosome segregation is a relatively passive process in *S. pneumoniae*. In line with this hypothesis, we found that transcription, one such ‘passive’ process, contributes significantly to efficient chromosome segregation. By perturbing the transcription machinery using antibiotics or mutating transcription factors, we observed a transcription-specific chromosome segregation defect. Finally, the combined deletion of the conserved transcription elongation factor greA and smc was synthetically lethal in *S. pneumoniae*, further demonstrating that efficient transcription is a driver of chromosome segregation in this oval-shaped bacterium.

**SW01.S1–12**
Hot spots of DNA double-strand breaks coupled with PARP1 and HNRNPA2B1 binding sites shape coordinately expressed domains in human chromosomes

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We observed DNA domains (known as forum domains) mainly separating in the region from 50 to 250 kb in pulsed-field gels. Domains are excised by spontaneous fragmentation of chromosomes from different eukaryotes (Tchurikov et al., PNAS, 1992). Now we developed a method for genome-wide mapping of DNA double-strand breaks (DSBs) that are responsible for this pulverization of human genome. Using 454 and Illumina deep sequencing platforms we mapped the hot spots of DSBs in human chromosomes. Analysis of genome-wide mRNA expression profiles in forum domains delimited by these hot spots of DSBs in several cell lines revealed that the genes located in the same domains are expressed much more coordinately than in the random stretches of the same number of genes in the genome. The usage of the circular permutation approach for genome-wide analysis (Cabrera et al., G3, 2012) of expression levels of genes in forum domains strongly argues in favor of the coordinated expression of genes in the same forum domain. In experiments in *vitro* and *in vivo* we detected the specific binding of PARP1 and HNRNPA2B1 at the borders of forum domains. Our data suggest the presence of a novel type of gene regulation: a coordinated silencing or transcription of gene clusters within large chromosomal domains that are protected from fragmentation, delimited by non-randomly dispersed DSB hot spots and the binding sites of PARP1.

**SW01.S1–13**
Whole-genome identification of somatic retroelement insertions in human brain tissues

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Retroelements comprise more than 40% of the human genome and are often considered to be ‘junk’ sequences. The vast majority of human retroelements have acquired many mutations that render them silent and are thus not able to transpose. However, some evolutionary youngest retroelements are still active in human genome. Their activity may lead to local genome rearrangements and alteration of gene expression. Recently it has been demonstrated that retroelements can amplify in human and murine neural tissues.

Our major goal was the whole-genome search for *de novo* insertions of transpositionally active L1HS and AluY retroelements in DNA from somatic neural tissues. We have developed a method of the active retroelements’ flanking sequences retrieval from genomic DNA, based on several consecutive stages of selective suppression PCR. High-throughput sequencing of the DNA libraries was carried out using the Illumina HiSeq system. The obtained sequences were mapped to the human genome. The identified coordinates were compared to the coordinates of the previously known insertions. We have analysed DNA from five tissues (cerebellum, cortex, subventricular zone of the left ventricle, dentate gyrus and myocardiun) of a single individual. The insertions detected in only one of the tissues were regarded as potentially somatic. The highest number of potential somatic insertions was detected in the cortex and in the subventricular zone. Some of the detected *de novo* insertions were verified by genomic PCR and Sanger sequencing.

**SW01.S1–14**
Tandem repeats of mouse and primate genomes *in silico* and *in situ*

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Much of tandem repeats (TR) functional nature in any genome remains enigmatic because there are only few tools available for dissecting and elucidating the functions of repeated DNA. We did a genome wide analysis of the large TR found in the mouse genome assemblies. Using a bioinformatics approach, TR arrays...
with monomer size up to 2 kb and array size more than 3 kb were identified in two mouse whole genome shotgun (WGS) assemblies. TR were classified into four superfamilies, eight families, and 62 subfamilies including 60 not previously described. The classification based on sequence similarity, chromosome position, monomer length, array variability, and GC content. The possibility of the existence of chromosome-specific TR had been predicted for mouse but no reliable cytogenetic probes were available before. We mapped 10 TR from different families on karyotyped metaphase plates. One mouse chromosome-specific TR found in silico and confirmed in situ; the rest of the chromosome-specific TR found did not exist in the assembled part of the genome. Four TR with strict subtelomeric location have been found. Annotated families of TR can be the source of cytogenetic probes for chromosome recognition.

TR of the 12 primate genomes, including *H. sapience*, have been analyzed with the rules and classification system used for mouse. The TR organization in different primates’ genomes are similar: the main TR families are conservative and there are few of them; but in each genome there are multiple species-specific TR subfamilies. The main TR families of different species possess similar motives in the monomers. It looks like our finding gives evidence for the ‘library hypothesis’ of the TR distribution in evolution.

During TR analysis of mouse and primate genomes it becomes visible that each chromosome possesses kind of unique code made up of different TR. The reference genomes allows to mark only internal TR and even with such a limited data the colored bar-code could be the carrier of the genome structural information, i.e. the order of precise TR association is the DNA morphogenetic program. Tandem repeats are the cores of the distinct 3D structures postulated in ‘gene gating’ hypothesis.

**SW01.S1–16**

**The role of microRNA-30c-2* as an anti-angiogenic mediator**

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**Purpose:** MicroRNAs (miRs) are small noncoding RNAs which negatively regulate the expression of targeted mRNA transcripts. Some miRs are already known to modulate angiogenesis. The p75 neurotrophin receptor (p75NTR) is upregulated in endothelial cells (ECs) of diabetic models and contributes to impaired post-ischaemic reparative angiogenesis in diabetes. To elucidate the miR signature of p75NTR in ECs, human umbilical vein ECs (HUVECs) infected with Ad.p75NTR demonstrated the upregulation of miR-30c-2* on an array. We aim to characterize the impact of miR-30c-2* on EC function.

**Methods:** Array results were confirmed by q-PCR using validated miR primers and SuU6 as an internal reference. To mimic advanced diabetes, where hyperglycaemia is accompanied by tissue starvation, HUVECs were cultured in high D-glucose (HG, 25 mM) and low growth factors (LG). For modulating miR expression, HUVECs were transfected with pre-miR precursor, anti-miR inhibitor or negative control. Transfected cells were prompted in proliferation (BrDU incorporation) and in vitro angiogenesis on Matrigel assays. Predicted miR target genes were searched using the databases Microcosm and TargetScan. Finally, relative miR-expression was quantified in limb muscles of type 2 diabetic db/db mice and non-diabetic controls. Statistical analyses are performed using either an unpaired t-test (two groups) or ANOVA (more than two groups).

**Results:** Relative expression of miR-30c-2* was upregulated (three-fold) in p75NTR-transduced HUVECs (p < 0.01 versus null-HUVECs) and five-fold in HG/LGF compared to culture in normal conditions (p < 0.01). Overexpression of miR-30c-2* in HUVECs impaired both proliferation and angiogenesis (40% decrease in proliferation and 25% decrease in tube length of Matrigel, p < 0.01 for both). In addition, miR-30c-2* was upregulated three-fold in the adductor muscles of db/db diabetic mice (p < 0.05 versus non-diabetic controls). A bioinformatics analysis identified the cell cycle regulator minichromosome maintenance complex component 7 (MCM7) as a putative miR-30c-2* target. In line, MCM-7 mRNA levels were decreased in p75NTR–HUVECs (by 57% versus Null-HUVECs, p < 0.05) and in HUVECs cultured in HG/LGF. Overexpression of miR-30c-2* also reduced MCM7 mRNA (by 64%, p < 0.01).

**Conclusion:** Our data suggest that the p75NTR-induced miRNA miR-30c-2* may act as an anti-angiogenic mediator in the context of diabetes by inhibiting the cell cycle regulator MCM7. miR-30c-2* may prove to be a novel therapeutic target for diabetes-induced impairment of post-ischaemic reparative neovascularisation.

**SW01.S1–15**

**Interaction between the nuclear matrix protein EAST and proteins of the Su(Hw) insulator complex in *Drosophila melanogaster***

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The best-characterized Drosophila insulator found in the gypsy retrotransposon contains 12 binding sites for the Su(Hw) protein. Along with Su(Hw), enhancer/silencer blocking requires Mod(mdg4)-67.2, a BTB/POZ domain protein. In particular, inactivation of Mod(mdg4)-67.2 leads to direct repression of the yellow gene promoter by the gypsy insulator. Previously we describe several su(mg) (suppressor of mod(mdg4)) genes mutations, which in the mod(mdg4) background, attenuated yellow repression induced by the gypsy insulator. One of these genes encodes EAST protein, a component of nuclear matrix. We demonstrate that repression induced by the gypsy insulator is regulated by the level of EAST and C terminal region of EAST play critical role in such repression. We found that EAST repression is mediated independently by insulator and LTR (long terminal repeat) sequences of gypsy transposon. The long terminal repeat of gypsy enhances the effect of EAST, suggesting that it contains an element interacting with the nucleoskeleton. Moreover we demonstrate direct interaction of EAST protein with general components of SuHw-depended insulator complex –Mod(mdg4)-67.2 and CP190 proteins. We found that level of EAST protein regulates assembly of insulator complex and distribution of insulator bodies in nuclei. These results first demonstrate the functional role of nuclear matrix in regulating insulator activity.
A novel method to calculate transcription factor binding in chromatin

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One of the main goals in quantitative biology is to predict gene expression from the assumptions of competitive and combinatorial binding of multiple proteins at genomic regulatory regions. This goal can be achieved with the help of computational algorithms that involve predicting gene expression from transcription factor (TF) binding maps. The main idea of such algorithms is it to consider the DNA sequence as a one-dimensional lattice with units of one or more base pairs [1]. To calculate protein occupancies in chromatin, one needs to take into account the competition of TFs and histone octamers for binding sites as well as the partial unwrapping of nucleosomal DNA [2]. This task is complex and computationally demanding, especially for genome-wide calculation. Our recent study has proposed a fast dynamic programming algorithm for the calculation of TF binding maps for eukaryotic cis-regulatory regions considering explicitly chromatin organization [3]. The initial parameterization of the proposed approach was performed on the basis of our data with respect to mouse embryonic stem cell development [4]. The method then has been applied to the calculation of TF binding maps for different types of mouse cells for which nucleosome positions have been determined experimentally. We have demonstrated how genome-wide changes of nucleosome positions in mouse cells alter TF binding, and showed that TF concentrations and nucleosomal DNA unwrapping influence the prediction of TFs binding sites.

References

Epigenetic aspects of HP1 exchange kinetics in apoptotic chromatin

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Apoptotic bodies are the most condensed form of chromatin. In general, chromatin structure and function are mostly dictated by histone post-translational modifications. Thus, we have analyzed the histone signature in apoptotic cells, characterized by pronounced chromatin condensation. Here, H2B mono-acetylation, and H3K9 and H4 acetylation was significantly decreased in apoptotic cells, which maintained a high level of H3K9 methylation. This phenotype was independent of p53 function and distinct levels of anti-apoptotic Bcl2 protein. Interestingly, after etoposide treatment of leukemia and multiple myeloma cells, H3K9 and H4 hypoacetylation was accompanied by increased H3K9me2, but not H3K9me1 or H3K9me3. In adherent mouse fibroblasts, a high level of H3K9me3 and histone deacetylation in apoptotic bodies was likely responsible for the pronounced (approximately 40%) recovery of GYP-HP1α and GYP-HP1β after photobleaching. HP1 mobility in apoptotic cells appeared to be unique because limited exchange after photobleaching was observed for other epigenetically important proteins, including GYP-JMD2 histone demethylase (approximately 10% fluorescence recovery) or Polycomb group-related GYP-BMI1 protein (approximately 20% fluorescence recovery). These findings imply a novel fact that only certain subset of proteins in apoptotic bodies is dynamic.

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Nuclear pattern and kinetics of HP1β protein

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Heterochromatin protein 1 (HP1) sub-types are versatile proteins preferentially involved in gene silencing and heterochromatinization. Moreover, HP1α, HP1β, and HP1γ were found to be recruited to UV-damaged chromatin, which is independent on methylation of histone H3 at the position of lysine 9 (H3K9me) that otherwise represents a binding site for HP1.1

Here, we focused on nuclear pattern and function of HP1β and we determined how deficiency of histone methyltransferases (HMTs) SUV39 h and inhibition of histone deacetylases (HDACs) influence the status of this heterochromatin-related protein HP1, H3K9me2, and H3K9me3 at clusters of centromeric heterochromatin and in compartment of nucleoli. We observed association of HP1β with both the chromocenters and fibrillarin-positive regions of nucleoli. Deficiency of SUV39 h and/or HDACi decreased the level of HP1β and H3K9me2 at chromocenters, but not in fibrillarin-positive regions of nucleoli that co-localized with RNA polymerase I. Based on these results, we suggested that the occurrence of HP1β in nucleoli is likely connected with transcription activity of ribosomal genes.2 Moreover, in locally induced double strand breaks, HDACi abrogated recruitment of HP1β to DNA lesions.

We also studied the kinetics of HP1β using fluorescence recovery after photobleaching (FRAP) technique. The recovery kinetics of HP1β was studied from the view of transcription level of entire genome. We confirmed rapid fluorescence recovery kinetics of HP1β in euchromatin when compared with heterochromatized of chromocenters.4 However, the recovery time after photobleaching was similar for HP1β in euchromatin of entire genome and compartment of nucleoli. According to these results, it is evident that protein density and protein abundance in heterochromatin and euchromatin likely influence protein mobility. Intriguingly, we observed significant changes in the recovery time of HP1β in heterochromatin regions, when the cell were treated by inhibitor of RNA Pol I, actinomycin D.5

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References
SW01.S1–20
1A2 insulator can interact with promoter of hsp70 gene in Drosophila melanogaster

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Insulators are regulatory DNA elements that participate in the modulation of the interactions between enhancers and promoters. Depending on the situation, insulators can either stabilize or destroy the contacts between enhancers and promoters. A possible explanation for the activity of insulators is their ability to directly interact with gene promoters. In the present study, it was demonstrated that, in model systems, a 1A2 insulator could interact with the core sequence of an hsp70 promoter. In this case, the insulator protein CP190 is found on the hsp70 promoter, which depends on the presence of an insulator in the transgene. The data obtained are consistent with the model, which implies that direct contacts between insulators and promoters make a considerable contribution to the modulation of the interactions between insulators and promoters.

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SW01.S1–21
A novel proteomic approach to study epigenetic changes in chromatin

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Different combinations of histone PTMs recruit different interaction partners, such as chromatin regulators. Development of new methodologies that allow one to study protein-protein proximity in vivo may provide important information about protein dynamics and help to decipher remarkable complexity of histone crosstalk.

We propose a variation of earlier developed proximity utilizing biotinylation (PUB) technique that is based on biotinylation of a protein fused to a biotin-acceptor peptide (BAP) by a biotin-ligase BirA, fused to its interaction partner. Fusion proteins containing this BAP are co-expressed with BirA in eukaryotic cells, leading to the specific biotinylation of the fusion protein. The new method is called PUB-NChIP (proximity utilizing biotinylation with native ChIP). PUB-NChIP is in vivo biotinylation approach to study chromatin in proximity to a protein of interest in which a nuclear protein of interest is fused to BirA and co-expressed with a BAP tagged histone. In a model experiment PUB was fused to specific histones and the ligase to Rad18, an E3 ubiquitin protein ligase associated with DNA repair. Biotinylation of specific histones in proximity to the Rad18 protein was observed. Biotinylation was used to isolate DNA associated with the subpopulation of histones proximate to the protein of interest. This method, similar to native ChIP, avoids the cross-linking necessary for conventional ChIP since core histones are relatively tightly associated with DNA. This preserves the ability to analyze post-translational modifications on the histones since lysines are the most frequent targets of crosslinking during ChIP and also common sites of posttranslational modification.

advantages of PUB-NChIP include the ability to utilize histone variants associated with specific functional states (e.g., active or repressed chromatin), and the possibility to perform pulse-chase experiments to monitor chromatin fate after it was in proximity with the nuclear protein of interest.

The application of the new methodology to study histone PTMs, developed in our work, may shed a new light on cellular mechanisms of chromatin remodeling, and have a general relevance for our understanding of the mechanisms of base excision repair and epigenetic reprogramming.

SW01.S1–22
Unique polymorphism of the gene CYP21A2 encoding the 21-hydroxylase in female patients with the signs of hyperandrogenism: data of the whole gene sequencing

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Hyperandrogenism is a pathology characterized by a high level of male sex hormones. One of the main reasons of hyperandrogenism is the autosomal recessive disease, congenital adrenal hyperplasia (CAH). More than 90% of CAH appear due to deficiency of the steroid 21-hydroxylase which is required for the conversion of progesterone to 11-deoxy cortisol and 17-hydroxyprogesterone to 11-deoxycortisol. Various mutations of the gene encoding this enzyme are the main reason of the reduced activity of the steroid 21-hydroxylase. Recently we have developed a DNA diagnostic system based on the allele-specific real-time PCR for detection of nine most common mutations which cause CAH (seven point mutations (SNP), and 8- and approximately 30-kb deletions). We made the DNA diagnostics of 15 patients with clinical and biochemical signs of hyperandrogenism. As a result, only one heterozygous mutation was found: the nonsense mutation 318GlnX (rs7755898). To characterize in more details the CYP21A2 gene of 15 patients mentioned above and 17 healthy women of control group, the 3293 bp fragments of genomic DNA (obtained from peripheral venous blood), which include whole CYP21A2 gene, were amplified by PCR and sequenced by Sanger method. Altogether 30 polymorphisms (28 SNP, one two-base substitution, and one trinucleotide insertion) were discovered, one of which was not earlier described. In four women from the control group were found common heterozygous mutations, which damage 21-hydroxylase: (two nonsense mutation 318GlnX (rs7755898), one missense mutation V281L (rs41315836), and one missense mutation P453S (rs6445). Six SNP (rs6450, rs6451, rs59064806, rs6543, and rs35147842), which localized in intron 2 and are typical for pseudogene CYP21A1P, as well as new SNP of exon 8, were not reviled in women of control group but were presented in five of 15 patients with signs of hyperandrogenism. The gene of each patient had a unique combination of polymorphisms inherent only in this very woman. As a result, we developed a model, which explains a possible drop in the activity of 21-hydroxylase in patients with the signs of the hyperandrogenism. We proposed that clinically neutral SNPs in the unique combinations can affect either the spatial structure of CYP21A2 mRNA or the efficiency of pre-mRNA splicing and decrease the level of gene expression. The fact might result in the development of the pathological phenotype in the patients.
Abstracts

SW01.S1–23
Laccase gene families in basidiomycetes from different taxonomy groups
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Fungal laccases are suggested to take part in lignocellulose degradation, soil organic matter cycling, stress response on diverse environmental challenges, etc. During the different stages of the fungal life cycle, laccase genes regulation and transcription is changed due to different environmental conditions and therefore differential expression of laccases isoenzymes implies alternative functions of these enzymes. However, the relationship between transcription and regulation of laccase genes and their expression remains unclear. The aim of the present study was the analysis of transcription level of genes encoded laccases from different white-rot fungi on glucose-peptone media (G-P) that commonly used for laccases production.

We studied 14 strains of white-rot fungi namely Antrodia sp., Byssomerulius sp., Coriolopsis sp., Peniophora sp., Pleurotus sp., Lenzites sp., Stecherinum sp., Trametes sp. and Xerula sp. The strains selected possessed high oxidase activity (spot-test with guaiacol and syringaldazine). Based on sequence analyses of ITS1 and ITS2 regions, all fungal strains were identified and the phylogenetic analysis has been carried out.

The laccase activities were detected in cultural broth of all strains except Byssomerulius and Xerula under submerged cultivation on G-P media. The 2D analysis revealed the presence of different laccase isoenzymes/isoforms in the fungi secretomes. However, neither laccase activity nor laccase isoenzymes/isoforms were detected in cultural broth of Byssomerulius and Xerula. In genomes of all fungi studied the multifamily laccase genes were identified by 454 technology (with exception of Peniophora and Xerula, where only one gene per strain was revealed). The amino acid sequences of laccases were determined. The complex phylogenetic analysis based on ITS analyses, the laccase amino acids sequences alignment and actin and β-tubulin sequences has been carried out. The screening of assembled contigs allowed to reveal the difference in the transcriptional levels of laccase gene family in each strain. As a result no correlation between the transcriptional level of laccases gene family and production of laccases encoded by these genes were found.

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SW01.S1–24
Structure and dynamics of a highly stable G-quadruplex with one imperfect G-tetrad
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G-quadruplexes (GQs) are non-canonical nucleic acid structures comprised of two or more G4-tetrads. GQs attract much interest with respect to their putative regulatory role in eukaryotic genomes. A highly stable GQ formed by a short oligonucleotide from CTIF gene (CTIF ON) is described. It contains a mismatch (G substitution for T) in one of the tetrads and thus represents an example of a new type of GQs – the so-called ‘imperfect GQ’. The fact that imperfect GQs can be stable under physiological conditions is of significant importance because ON sequences forming such structures cannot be predicted by the currently available

GQ-search algorithms. The number of stable non-canonical structures in genomes may thus be underestimated and the GQ-search algorithms need to be improved. CTIF ON was shown to adopt a monomolecular parallel GQ structure (CTIF GQ) under physiological conditions by physicochemical methods (CD and UV-melting). Available GQ-search algorithms suggest that CTIF ON can only fold into a two-tetrad GQ. However, our NMR results suggest that 14 G residues of CTIF ON participate in G-tetrad formation, which implies the existence of three perfect tetrads and one mismatched one. To clarify this, we modeled CTIF GQ using the XDR data on its structural analogs, two known parallel 4-tetrad GQs. Two CTIF GQ models differing in the twist of ON strands were built and optimized by 15 ns molecular dynamics simulation. During the simulation, one of the model structures underwent a rearrangement: the mismatched nucleoside was displaced from the internal tetrad by the G residue from the adjacent external tetrad. Free energies of the optimized model structures were evaluated and compared. The results of our study suggest that GQs with imperfect tetrads can be thermodynamically stable and the mismatches are better tolerated in external tetrads.

SW01.S1–25
A study of insulator-promoter interactions in Drosophila
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Chromatin insulators are special regulatory elements involved in modulation of enhancer–promoter communication. Drosophila yellow and white genes contain insulators located immediately downstream of them, I2A and Wari, respectively. Using an assay based on the yeast GAL4 activator, we have found that both insulators are able to interact with their target promoters in transgenic lines, forming gene loops. The existence of an insulator–promoter loop is confirmed by the fact that insulator proteins could be detected on the promoter only in the presence of insulator in the transgene. The upstream promoter regions, which are required for long-distance stimulation by enhancers, are not essential for promoter–insulator interactions. Both insulators support basal activity of the yellow and white promoters in the eyes. Thus, the ability of insulators to interact with promoters can play an important role in regulation of basic gene transcription.

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SW01.S1–26
Transcription through enhancers suppresses their activity in Drosophila
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Enhancer elements determine the level of target gene transcription in a tissue specific manner, providing for individual patterns of gene expression in different cells. Knowledge of the mechanisms controlling enhancer action is crucial for understanding global regulation of transcription. In particular, enhancers are often localized within transcribed regions of the genome. Using a transgenic reporter system, we have shown that intergenic transcription affects the activity of different Drosophila enhancers, counteracting their ability to activate the target promoters. The efficiency of inhibition of enhancer action is directly proportional to the level of interfering transcription. As expected, a presence of a transcriptional terminator between the inhibiting promoter

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Akt1 mediated Hox gene expression through epigenetic modifications in mouse embryonic fibroblasts

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The evolutionarily conserved Hox genes are organized in clusters and expressed co-linearly to specify body patterning during embryonic development. Recently, in search of regulatory genes responsible for the co-linear expression of Hox genes, Akt1 has been identified as a putative Hox gene regulator from GEO dataset. Here we provide evidence that Akt1 regulates Hox gene expression by epigenetic modification. In this study, substantial upregulation of the 5′ Hoxc genes has been observed in Akt1−/− MEFs. Enrichment of histone H3K9 acetylation, an active histone marker, showed positive correlation with gene expression patterns. HDAC inhibitors de-repressed 5′ Hoxc gene expression in wild-type MEFs, and DNA demethylating reagent synergistically upregulated HDAC-induced 5′ Hoxc gene expression. In Akt1 null MEFs, Gcn5 inhibitor, MB-3 repressed Hoxc11 gene expression, and consistently, RNA interference inhibition of Gcn5 led to repressed Hoxc11 gene level. The absence of Akt1 increased Gcn5 protein stability and protein expression. On the other hand, enrichment of histone H3K27 trimethylation, a repressive histone marker, showed a negative correlation with 5′ Hoxc expression patterns. The expression levels of Utx and Jmjd3 histone H3K27me3 demethylases were upregulated in Akt1 null MEFs and moreover, knockdown of Akt1 induced Utx and Jmjd3 expression, leading to the reduction of H3K27 trimethylation. The repression of 5′ Hox expression by Akt1 requires the function of histone deacetylases. In Akt1 null condition, histone acetyltransferase and demethylase induce derepression of 5′ Hoxc genes through histone H3K9 acetylation and H3K27me3 demethylation on gene promoter regions. Taken together, these results suggest that Akt1 is essential for the epigenetic regulation of Hox genes. This work was supported by a grant 2010-0026759 from National Research Foundation and partly by the BioGreen21 Program (PJ00905601) of RDA, Korea.

G-Quadruplexes with imperfect tetrads are stable under physiological conditions and may be prevalent in human genome

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Non-canonical polynucleotide structures are shown to play a key role in the processes of biogenesis, such as transcription, DNA repair, replication, translocation and RNA splicing (Saini, Zhang et al. 2013). Therefore, studies of the dynamics of DNA and RNA secondary structures are necessary for understanding the mechanisms of the genomic regulation and identifying new biomarkers of pathologies and drug targets.

Solution structures of single-stranded G-rich DNA fragments from the promoter region of human Bcl-2-gene and the intron of CTIF-gene, as well as a wide range of model oligonucleotides, have been investigated. Formation of unexpectedly stable quadruplexes with one or more imperfect tetrads (Imperfect G-quadruplexes, ImGQs) has been detected.

Our results provide insight into the diversity of non-canonical DNA structures. To assess the abundance of ImGQs in genomic sequences, an improved GQ-search algorithm and a corresponding program (ImGQfinder) were developed. The improved algorithm allowed us to identify more than 130 000 putative ImGQs in human genome in addition to 70 000 previously known ‘perfect’ putative GQs. The analysis of ImGQ location and distribution was performed on the 18th chromosome.

To sum up, G-quadruplexes with imperfect tetrads have been described and their stability in solution has been assessed. Together with the recently published data on a stable GQ with a single bulge (Mukundan and Phan 2013), our findings suggest that secondary structures adopted by G-rich DNA fragments are more diverse than previously thought.
SW01.S1–30
Common cell polyploidy-associated transcriptomic traits in evolutionary distant organisms
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Polyploid cells show great among-species and among-tissues diversity and relation to developmental mode, suggesting their importance in adaptive evolution and developmental programming. At the same time, excessive polyploidization is a hallmark of functional impairment, aging, growth disorders, and numerous pathologies including cancer and cardiac diseases. The data coming from various fields of research indicate that polyploidy exerts various effects in various cell types suggesting that its pure effects are still not completely understood. It is not surprising, because preserving gene dosage balance, genome doubling may induce only very subtle effects that are very difficult to filter from the noise. At the same time catching these effects is important, because many regulatory molecules, including transcription factors, are synthesized in a number of just several copies. Also it has been recently shown that even tiny perturbations in DNA specificity, affinity, and promoter design can transform an individual TFs between distinct roles and alter the signal processing behaviour of multi-input systems. To find out how genome duplication affect transcriptome activity, we compared the data from multiple databases obtained with next generation sequencing and whole genome microarray analysis on cells from evolutionary distant organisms including yeast and mammals. Using multi-faceted bioinformatic approach, we indicated that polyploidy activates protein synthesis and degradation through ubiquitin system, boosts energy metabolic pathways, particularly sugar and fatty acid metabolism, and triggers stress response to various stimuli. Thus, our results suggest cell polyploidy is a powerful instrument of epigenetic transcriptome regulation. A detailed description of ploidy-related transcriptomic changes is critical for our understanding of gene activity epigenetic control in health in disease because it may be helpful in the creation of new approaches to disease prevention and therapy.

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SW01.S1–32
Interplay between duplicated genomes in mammalian hepatocyte and cardiomyocyte
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Excessive somatic polyploidy is associated with the most common and dangerous human diseases, including, hypertension, atherosclerosis, and cancer. At hypertension and atherosclerosis, excessive genomes were registered in cardiomyocytes, vascular smooth muscle cells and megacaryocytes. At breast, pancreatic, prostate, lung and colon carcinomas, cell tetraploidization is known to be an initial event preceding highly invasive aneuploid state. Thus, it is reasonable to suggest that somatic polyploidy may participate in adaptation to pathologic state and that it participates in genome functional anatomy changing. To elucidate the functional significance of genome multiplication in somatic tissues, we performed a genome-scale analysis of ploidy-associated transcriptomic changes in human and mouse heart and liver, which have the reciprocal pattern of polyploidization. For this purpose, we use the network analysis approach and genome-scale cross-species comparison. Our data indicate that heart and liver show similar traits in response to polyploidization. In both organs, polyploidy protects vitality, triggers cell renewal and pro-oncogenic pathways. Also we found that polyploidy induces reserve ATP production pathways and sustains tissue-specific functions by switching them to energy saving mode. We also found that polyploidy comes together with mild hypoxia-inducible changes, including cell survival pathways induction, sugar and fatty acid metabolism boosting and stress response activation. Thus, somatic polyploidy may be an ingenious evolutionary instrument for fast adaptation to stress and new environments allowing trade-offs between high functional demand, stress, and energy depletion. This study was supported by The Ministry of Education and Science of Russian Federation, project 8306, RFBR No. 12-04-01199-a, and, Russian Academy of Sciences presidium program 'Molecular function integration mechanisms for physiologic function realization'.

SW01.S1–31
Dual role of gammaH2AX in the cellular response to hyperthermia
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Heat shock (HS) is one of the better-studied exogenous stress factors. However, little is known about its effects on DNA integrity and the DNA replication process. In this study, we show that heat shock affects DNA structure and DNA-associated processes in a cell cycle phase-dependent manner. In G1 and G2 cells, HS induces a countable number of DNA double-stranded breaks (DSBs), which are marked by γH2AX. In contrast, in S-phase cells, HS does not induce DSBs but causes an arrest or deceleration of the progression of the replication forks in a temperature-dependent fashion. Interestingly, this response also provoked the phosphorylation of H2AX, which appeared at sites of replication (replication foci). Moreover, the results obtained suggested that the phosphorylation of H2AX at or close to a replication fork rescued the fork from total collapse. Collectively, our data suggest that in an asynchronous cell culture, heat shock might have a dual effect on the DNA integrity and that phosphorylation of H2AX has a directly protective effect on the arrested replication forks in addition to its known DSB signaling function.

SW01.S1–33
Identification of new Drosophila proteins involved in insulator functions
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dCTCF (homolog of vertebrate insulator protein CTCF) is one of the best studied Drosophila insulator proteins that contain DNA-binding domains consisting of multiple zinc-fingers. In transgenic assay, the dCTCF binding sites can support specific distance interactions suggesting a key role of these proteins in organization of chromatin architecture. dCTCF protein interacts with Centrosomal Protein 190 (CP190) that contains an N-termin al BTB/POZ; an aspartic-acid rich D-domain; four C2H2 zinc-finger motifs and a C-terminal E-rich domain. We identified two
new proteins that interact with CP190. Using the yeast two-hybrid system and GST pull-down we showed that the BTB and M domains of CP190 are required for interaction with these proteins. Role of these new proteins in insulation and long-distance interactions will be presented.

SW01.S1–34
Highly conserved Eny2/Sus1 protein binds to Drosophila and human CTCF and is required for barrier activity
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Chromatin insulators affect interactions between promoters and enhancers/silencers and function as barriers for spreading of repressive chromatin. Drosophila insulator protein dCTCF marks active promoters and many borders of histone H3K27 trimethylation domains associated with repressed chromatin. In particular, dCTCF binds to many boundaries located between the parasegment-specific regulatory domains of Bithorax-complex. Here we demonstrate that an evolutionarily conserved protein ENY2 is recruited to the zinc-finger domain of dCTCF and is required for barrier activity of all tested dCTCF-dependent promoters and insulators. Inactivation of dCTCF only partially reduces binding of ENY2 to chromatin suggesting that additional proteins are required for recruiting of ENY2. Inactivation of ENY2 by RNAi in Drosophila cells led to spreading of H3K27 trimethylation and Pc protein at all tested dCTCF boundaries. The human homologous CTCF and hENY2 were shown to interact in vitro suggesting that this protein-protein interaction is conserved in evolution. Based on the results of genetic and in vitro studies, we conclude that ENY2 is responsible for recruiting of evolutionary conserved non-identified complex that is essential for establishment of barrier between active and silencing chromatin domains.

SW01.S1–35
Functional analysis of a chromosomal regulatory element based on its addressed insertion into the primary transgene
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Transposable elements such as P elements have been important tools to introduce different engineered transgenic constructs into genome of Drosophila with a purpose to study genomic elements in vivo. Unfortunately, genomic elements in the vicinity of P element insertion sites often can interfere with the transgene functioning. Typically, many transgenic lines, having transgenes in different locations, should be obtained and investigated to make correct conclusion about the transgenic elements functioning. New combinatorial methods based on the insertion of the DNA of interest into particular convenient genomic address could make this work more efficient. Here, we describe one such method. We have combined P element-based insertion of a primary transgene into Drosophila genome with the following phiC31 integrase-based recombine-mediated cassette exchange (RMCE) of the dominant genetic body color marker (gene yellow) flanked with two recombination docking sites (attP) in the primary transgene for the hypothetical regulatory element (thrithorax response element, TRE, recently identified in the fork head distal promoter region in our lab). For the RMCE step we have used co-injec-

SW01.S1–36
Spider genes encoding two-domain toxins
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Spider venoms mostly contain peptide toxins belonging to two structural groups, linear toxins and cysteine-rich knottin (ICK) toxins. More recently the structural class of two-domain modular spider toxins has been recognized. These molecules are built from two modules, each showing structural similarity to single-domain peptides. Different combinations produce the following two-domain toxin subclasses: linear-linear, knottin-knottin, linear-knottin, and knottin-linear. Here we report cDNA and gene sequences encoding two-domain toxins of two subclasses, knottin-knottin (CpTx from Cheiracanthium punctatum), and linear-knottin (OtTx from Oxyopes lineatus). To date there is very limited information on the structure of spider toxin genes, and most of the studied sequences do not contain introns. Analysis of two-domain toxin genes revealed that they do not contain introns either.

We further discuss possible evolutionary scenarios of two-domain toxin emergence. The toxins from Oxyopes are chimeras of linear and knottin domains. We hypothesize that their 'mosaic' genes evolved from two different parental genes. Most likely they result from insertion of a sequence encoding the linear module into a parental knottin gene between the prosequence and mature chain fragments. In contrast, two-domain toxins from Cheiracanthium are built from two knottin modules. This might be the result of a gene duplication event, but amino acid sequences of the two domains show only moderate similarity. Sequences exhibiting higher similarity to the separate domains were found in cDNA library from venom glands of Lycosa singoriensis. We suppose that insertion of a sequence encoding the C-terminal domain occurred into the 3' region of an ancestral gene encoding the N-terminal domain.

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SW01.S1–37
Comet assay as a tool to investigate topology of DNA loops in intact cells
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In our experiments we measured the kinetics of comet formation in intact lymphocytes in neutral comet assay (single cell gel electrophoresis). Our results have shown that the comet tail is...
formed due to an extension of supercoiled DNA loops. This extension was reversible after switching off the electrophoresis provided that the DNA torsional constraints remained in the loops. The DNA exit is essentially hampered by the constraint: the comet formation is facilitated when the loops lose their supercoiling either upon nicks accumulation after an X-ray irradiation or due to local unwinding of the double helix upon intercalation [1]. In our experiments two intercalators – ethidium bromide (EtBr) and chloroquine (Chl) – were used. At particular concentrations of the intercalators (0.05 µg/ml for EtBr, 25 µg/ml for Chl) DNA loops were relaxed and the comet formation was mostly facilitated. At these concentrations the positive supercoiling density introduced by the intercalators equals to the initial negative supercoiling in absolute value. Further increase in the intercalator concentration introduced an accumulation of the positive supercoiling that again hampered the DNA exit. Using the intercalator concentration introduced an accumulation of the positive supercoiling in absolute value. Further increase in the intercalator concentration introduced an accumulation of the positive supercoiling that again hampered the DNA exit. Using the intercalator concentration introduced an accumulation of the positive supercoiling in absolute value. Further increase in the intercalator concentration introduced an accumulation of the positive supercoiling that again hampered the DNA exit.

Reference


SW01.S1–38
Nuclear translocation of myosin VI (MVI) due to cell stimulation in PC12 cells: a possible role of MVI in gene transcription

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Myosin VI (MVI) is a unique, actin-based motor protein that moves along actin filaments in the opposite direction of all the other classes of myosins. We have previously shown that in neurosecretory PC12 cells MVI is present both in the cytoplasm and in the nucleus, and that decrease of MVI expression caused inhibition of cell migration and proliferation. Here, we show that nuclear localization of MVI is even more pronounced due to cell stimulation with 56 mM KCl and incubation with leptomycin B, inhibitor of the nuclear export. The accumulation is associated with MVI colocalization within the nucleus with active RNA polymerase II, transcriptionally active sites (BrUTP incorporation assay) and transcription factor Sp1, as well as with several nuclear structures involved in nascent transcript maturation (PML bodies, nuclear speckles and hnRNP particles). In addition, mass spectrometry analysis of the PC12 cell extract obtained from a pull-down experiment with the C-terminal tail domain of MVI revealed several new potential MVI binding partners, among them proteins involved in transcription and post-transcriptional processes. These data indicate that stimulation-dependent increase of MVI in the nucleus is associated with the increase of transcriptional activity, thus supporting a notion that MVI could be involved in the regulation of gene expression.

SW01.S1–39
Intra- and interspecies evolution of beta-fructosidase SUC genes in the yeast Saccharomyces

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Sucrose is one of the major natural carbon source for Saccharomyces yeasts. Hydrolysis of this disaccharide may be processed by invertase enzyme (beta-fructosidase) encoded by polymeric genes SUC1-SUC5 and SUC7-SUC10, which (except for SUC2) are located in the subtelomeric regions of different chromosomes (Mortimer at al. 1992; Naumov & Naumova 2010). The SUC2 gene is obligatory present in S. cerevisiae and its six sibling species: S. arboricola, S. bayanus, S. cariocanus, S. kudriavzevii, S. mikatae and S. paradoxxus. Strains with multiple SUC genes are only found in S. cerevisiae distillery, bakery and brewery yeasts (Mortimer et al. 1992; Naumov et al. 1996). Using molecular karyotyping and Southern-hybridization we have compared genomes of 36 distiller's strains of S. cerevisiae. Southern hybridization with the SUC2 probe revealed considerable polymorphism of hybridization profiles among the strains. Accumulation of polymeric genes of sugar fermentation with cumulative effect in a single strain leads to intensification of the process (Hohmann 1987). Indeed, most of the strains studied by us, which were able to ferment sucrose rapidly, have several SUC genes. To infer molecular evolution of Saccharomyces beta-fructosidase SUC genes, we have sequenced SUC2 gene from S. arboricola and new SUC8-SUC10 genes from S. cerevisiae. The SUC sequences obtained were compared with the database SUC sequences of Saccharomyces yeasts. Comparisons of the SUC genes in all combinations revealed no deletions or insertions and showed that transitions are more numerous than transversions. Transitions C–T prevailed in the total spectrum of nucleotide substitutions and were mostly detected in the third codon position. Similar results were earlier reported for the SUC1, SUC2, and SUC4 genes (Hohmann, Gozalbo 1989). Most transitions in the third codon position are silent, causing no change in the amino acid sequence of the encoded protein. Therefore, the spectrum of nucleotide substitutions observed for the coding regions of the SUC genes may result from selection aimed at conservation of the amino acid sequence of beta-fructosidase. Phylogenetic analysis of the deduced amino acid sequences revealed the species specificity of beta-fructosidases in Saccharomyces yeasts.

SW01.S1–40
Chromosomal polymorphism of LAC genes for lactose fermentation in dairy probiotic yeasts Kluyveromyces

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Lactose fermentation of microorganisms, including the dairy yeasts Kluyveromyces lactis and K. marxianus, is a significant property for basic and applied studies. In particular, this feature is of importance for studying probiotic activity of Kluyveromyces yeasts. Polymy of closely linked LAC4 and LAC12 genes encoding beta-galactosidase and lactose permease in Kluyveromyces has been determined in our laboratory, for the first time
SW01 Mechanisms of Genetic Control

Abstracts

(Naumov 2008). We have conducted a large scale molecular-genetic screening of many Kluyveromyces strains, mainly of dairy origin. New polymeric loci LAC, carrying LAC4–LAC12 clusters have been determined in K. lactis and K. marxianus. Molecular karyotyping and Southern analysis revealed polymorphism of hybridization profiles among K. lactis yeasts isolated from different dairy products. Some strains contain several polymeric LAC loci. Along with the LAC1 and LAC2 (Herman, Halvorson 1963), a novel LAC3 locus was observed. Unlike natural non-fermenting or weakly fermenting lactose populations of Kluyveromyces yeasts, dairy strains can accumulate polymeric LAC loci in their genomes. The LAC genes located in subtelomeric regions of different chromosomes may have a cumulative effect. Taking into account the data obtained, we propose the following nomenclature of the LAC genes: (LAC4-LAC12/1, (LAC4-LAC12/2, (LAC4-LAC12)/3, (LAC4)/1, (LAC4)/2, (LAC4)/3, (LAC12)/1, (LAC12)/2 and (LAC12)/3. Scientific and applied significance of LAC genes is discussed.

SW01.S1–41

Low yield of 3C ligation products: technical issues or infrequent interaction between DNA regulatory elements?

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Chromosome conformation capture (3C) and derivative experimental procedures are used to estimate the spatial proximity between different genomic elements, thus providing information about the 3D organization of genomic domains and whole genomes within the nucleus. All C-methods are based on the proximity ligation – the preferential ligation of joined DNA fragments obtained upon restriction enzyme digestion of in vivo cross-linked chromatin. Here, using the mouse beta-globin genes in erythroid cells as a model, we estimated the actual frequencies of ligation between the fragments bearing the promoter of the major beta-globin gene and its distant enhancers and showed that the number of ligation products produced does not exceed 1% of all fragments subjected to the ligation. Although this low yield of 3C ligation products may be explained entirely by technical issues, it may as well reflect a low frequency of interaction between DNA regulatory elements in vivo.

SW01.S1–42

Long-term effects of cryptosporidial gastroenteritis on neonatal rat cardiomyocyte

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Developmental origin of health and diseases theory (DOHaD) postulates that growth retardation and inflammation in children increase cardiovascular pathology risk decades later. Based on medical statistics, suggesting that gastroenteritis is the most common cause of growth retardation and inflammation in children, we investigated the postponed effects of cryptosporidiosis challenged by the wide-spread human enteropathogen – Cryptosporidium parvum on neonatal rat cardiomyocyte ploidy and myosin heavy chain expression. Our results suggest that moderate neonatal cryptosporidiosis triggers long-term cardiomyocyte pathology. Using real-time PCR, immunocytochemistry and image analysis, we demonstrated that 2 and 6 month-old rats who survived cryptosporidiosis at the age of 10–14 days, showed cardiomyocyte hyperpolyploidization and myosin heavy chain expression balance shift toward the slow isoform beta. Unexpectedly, animals who survived the disease 5.5 month ago, showed more prominent changes than the ones who survived cryptosporidiosis 1.5 month ago. This data suggest that cryptosporidiosis is particularly dangerous for weakened or ageing organism. Based on the observation that myosin heavy chain β possesses approximately five times lower ATP-ase activity compared to myosin heavy chain α, it is reasonably to suggest that neonatal cryptosporidiosis decreases cardiac contractile ability and thus may trigger developmental programming of adult cardiovascular diseases.

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SW01.S1–43

Transcriptional activity of superoxide dismutase genes in aphid-stressed maize seedlings

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The objective of this report was aimed at assessing influence of bird cherry-oat aphid (Rhopalosiphum padi L.) infestation on the transcriptional activity of sodA, sodB and sodA genes encoding superoxide dismutase isozymes (mitochondrial Mn-SOD and two cytosolic isoforms of Cu/Zn-SOD) in maize. The experiments were performed on 8-day-old seedlings of two maize cultivars (Tasty sweet – susceptible and Ambroza – relatively resistant) that were artificially infested by 5, 10, 20 and 40 apterus adult aphids per plant. The control group of seedlings was non-infested by the insects. Molecular responses in transcript levels of the studied genes within aphid-stressed Z. mays plants were analysed at 1, 2, 4, 8, 24 and 48 h post initial infestation (hpi). The qRT-PCR technique was used in order to determine the relative expression of the targeted genes in maize tissues (GAPDH, glyceraldehyde 3-phosphate dehydrogenase gene was used as an internal control). Aphid colonization resulted in a significant enhancement in the transcript levels of all examined sod genes in maize seedlings of both tested genotypes. Time-course analysis revealed that a maximal increase in the relative expression of sod3.4 and sod4 genes within stressed Z. mays seedlings was reached at 24 hpi, whereas the highest accumulation of sod4A transcript was noted at 24 h of aphid infestation. It should be underlined that aphid-injured plants of R. padi-resistant Ambroza cv. reached significantly higher gene expression levels when compared to R. padi-susceptible Tasty sweet cv. The obtained results support the hypothesis that superoxide dismutase contribute to limiting deleterious effects of excessive amounts of reactive oxygen species generated in aphid-stressed maize seedlings.

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SW01.S1–44

Biological functions of linker histones in Arabidopsis

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Linker histones (H1) are ubiquitous and conserved structural proteins occurring in eukaryotic chromosomes. In both animals and plants there are multiple non-allelic variants of H1 which differ in chromatin binding properties and have been suggested to take part in control of genetic programs during development and differentiation. Arabidopsis thaliana, a widely used model organism in plant biology, has three non-allelic variants of H1 (linker) histones: H1.1, H1.2 and H1.3. It has been suggested that Arabidopsis H1s may affect gene expression by regulating the specificity of DNA methylation [1]. Here, we analyzed Arabidopsis lines with mutations in one, two or all three variants of H1 in order to assess their contribution to growth and developmental transitions in Arabidopsis as well as their role in occurrence and distribution of various epigenetic marks in chromatin. We discuss the results in the broader context of the chromatin function of histone H1.

Reference

SW01.S1–45

A multipotent zinc finger protein essential for wing development in Drosophila melanogaster

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The C2H2 zinc finger gene family of transcription factors is the most predominant and abundant class of transcription factors in eukaryotes. The members of the family participate in fundamental mechanisms of gene expression. However, in accordance with their diverse structures, they also play more specific roles in a wide variety of regulated biological processes including signal transduction, cell growth, differentiation and development.

The zinc finger protein Vfl, a key activator of the early zygotic genome in Drosophila melanogaster, is shown to be necessary for patterning the wing structure during late developmental stages of the fly. This protein has also been shown to play crucial roles during embryogenesis such as sex determination and neurogenesis. In vivo experiments have shown that knockdown or overexpression of Vfl gene’s products in larval wing disc regions result in abnormal patterning of adult wing tissue. The phenotypes observed range from mild notching of the adult wing to its complete absence. Furthermore, our data suggest that it can affect major components of signalling pathways that govern wing development. The transcriptional targets as well as the exact mechanism of Vfl’s involvement in patterning decisions is subject to further investigation.

SW01.S1–46

Telomere length between mononuclear blood cells (MNC) and peripheral white blood cells (WBC) in context with population-specific mitochondrial (MT) lineages in a Latvian population ageing

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Previous studies have reported that telomere length in MNC is longer than in WBC. Population studies have demonstrated that telomere length in one age group can be quite diverse in different European populations and the Baltic region. There were no studies performed on the variability of telomere length in an aging Latvian population in association with population-specific MT lineages. The aims of this study were to observe any variation in telomere length in different age groups and evaluate the dynamics of telomere length with MT genetic background.

Samples from 121 individuals (20–100 years old) were collected. MNC were obtained from 21 samples using ACCUSPIN System-HISTOPAQUE-1077 tubes. Telomere length was detected using the TeloTAGGG Telomere Length Assay kit. Statistical analyses – GraphPad Prism 5 Software.

The results demonstrated that telomeres from MNC were longer than telomeres from WBC by 11.5%, (difference approximately 822 bp, p < 0.0001). Comparison of telomeres from these cell types in two different age groups, i.e., the control group (20–40 years) and the second group (65–85 years), suggested that after 65 years of age telomere length decreases by a smaller degree in MNC than in WBC (p = 0.1890). Comparing telomere length in MNC versus WBC between the control group (20–40 years) and the second group (65–85 years), the results showed difference in MNC telomere length by age group (p = 0.0428), but there was no significant difference in WBC telomere length by age group.

Our preliminary results showed a tendency that the frequency of haplogroup (hg) H gradually decreased with age. In contrary, hg U did not demonstrate the same pattern. Likewise, comparing both hg (H and U), individuals harboring hg H displayed the gradual decrease of telomere length with age. Moreover, hg U showed fluctuations in different age groups. The most abundant haplogroups among Latvians are H and U, and comprise approximately 70% from total MT lineages. Therefore, the population structure of MT gene pool may affect mechanisms of telomeres shortening. Dynamics of telomere length in WBC and MNC cells from different age groups indicated on different patterns in a population under the study.

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SW01.S1–47

Analysis of epigenetic pathways of enhancer and insulator functioning in genetic constructs transfected into Drosophila S2 cells

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Enhancer and insulator are the key regulators of gene expression. We elaborated a system using transfection of genetic constructs, excluding the influence of other regulatory elements in cis on epigenetic states in different regions of the constructs. Previously we...
detected in *Drosophila melanogaster* cultured S2 cells a promoter-independent synthesis of non-coding RNAs in the intergenic regions of the constructs induced by enhancer (enhancer RNAs, eRNAs). The synthesis was induced by the enhancer from mobile element *copia* in both directions and repressed by a single *gypsy* insulator or by a pair of the insulators.

Now using 5′ RACE and 454 deep sequencing and Nothern-blot analysis approaches we detected that the most part of transcription start sites (TSS) of 300–1050 nt eRNAs are located at 250 bp distance from the enhancer. In this study, we also revealed, that enhancers induce the H3K4me3 and H3K18ac modifications in the regions of eRNA synthesis. We observed that insulators act as antagonists of enhancers, because insulators reduce both the levels of eRNAs synthesis and H3K4me3 and H3K18ac marks produced by enhancers, and at the same time induce H3K4me1 and H3K27ac marks. We also found that insulator proteins bind in different regions of genetic constructs. e.g., induce H3K4me1 and H3K27ac marks. We also found that insulator or by a pair of the insulators.

In this study, we also revealed that pan-neural and muscular RNAi in *Drosophila* revealed that the genomic copies of *Lim3A* transcribed with a sixfold change in the *Lim3A* expression and a 25% change in *Drosophila* lifespan. This region was found to be very conservative throughout *Drosophila melanogaster* group and comprised sites for Polycomb/Trithorax-group proteins. Using dual luciferase reporter assay, we revealed *Lim3A* to be TATA-less DEP-containing gene. We also found out a 680 bp long DNA fragment located upstream of the *Lim3A* TSS was sufficient to provide maximum *Lim3A* expression in S2 cells. Deletion of the regulatory element located within 380–680 bp upstream of the *Lim3A* TSS resulted in three fold decrease in gene expression.

We conclude that the DNA region located within 380–680 bp upstream of the *Lim3A* TSS is sufficient for the *Lim3* transcription regulation. We hypothesize that regulation of *Lim3* transcription and subsequent life span alterations can be mediated by Polycomb/Trithorax-group proteins.

**SW01.S1–49**

**De novo assembly and preliminary annotation**

*Rhytididiadelphus squarrosus* (Bryophyta) large-scale transcriptome data

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Transcriptomics is one of the fastest growing area of functional genomics, the study of the RNA transcripts structure is the key to understanding the functional genome organization in different representatives of the organic world. Unfortunately, this is crucial fundamental and applied problem is far from its solving, only for very restricted numbers of organisms some large-scale transcriptome data are available now. Liverworts (Marchantiophyta) and mosses (Bryophyta) are the most ancient groups of higher plants diverged more than 450 mya and developed the earliest evolutionary adaptations for terrestrial way of living. This is why their genome studies deserve special attention. In our research approximately 2 µg of total RNA was extracted from axenic culture of *Rhytididiadelphus squarrosus* (Hypnales, Bryophyta) and after cDNA synthesis its sequencing was performed on NGS platform Illumina GAIIx. Both library preparation and sequencing procedure were performed following standard Illumina protocols. The raw sequencing data was comprised of 53,390,473 pairs of 76 nt reads. Various read pre-processing strategies were applied. Hereafter Oases and Trinity assemblies were accomplished. The total length of transcripts varied from 76,486,645 nt (Trinity assembly) to 655,870,074 nt (Oases assembly). The total number of transcripts also was strongly dependent on applied assemblers (67,145 transcripts for Trinity and 484,078 for Oases assemblies, respectively). The maximal transcripts size was 163,09 nt for Oases and 143,297 nt for Trinity. On the other hand, the maximal number of unique loci was in Trinity assembly (41,978 against 30,615 in Oases assembly). For functional annotation and analysis of transcripts Blast2GO tool was applied to Trinity data. By now only part of assembled contigs was analyzed. From 300,000 recovered Blast hits16% were blasted against *Physcomitrella patens*, 73% – against 28 other higher plant species, and 11% – against other organisms. Among 15,048 annotated sequences 830 are associated with cytoplasm, 448 with plastids, 208 with mitochondria, and 264 with nucleus. Other Blast statistics and GO annotations were recovered.

**SW01.S1–48**

**The transcriptional activity of neuronal genes in lifespan control: mechanisms regulating transcription of *Drosophila melanogaster* Lim3 gene**

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Senescence is a process which is characterized by substantial variation within and across species. This variation can be provided by environmental and genetic factors. Indeed, the expression alteration of many genes was shown to affect lifespan in different organisms. It is reasonable to assume that an alteration of expression of some master genes, such as genes encoding transcription factors which affect the expression of many target genes could be especially crucial for lifespan control.

Previously, using different genetic approaches, we identified several candidate genes that affect *Drosophila melanogaster* lifespan. One of these genes, *Lim3*, encodes an RNA polymerase II transcription factor which is highly homologous to human LHX3/4 proteins. *Lim3A* transcript of this gene was previously found to be essential for *Drosophila* neuron development. We revealed that pan-neural and muscular RNAI in *Drosophila* lines against *Lim3* gene in early development affects early survival rates of *Drosophila* and *Drosophila* locomotion behavior.

We found out that naturally occurring molecular polymorphism in the DNA region located within 380–680 bp upstream of the *Lim3A* transcription start site (TSS) was significantly associated with a sixfold change in the *Lim3A* expression and a 25%...
SW01.S1–50

Characterization of long range interactions of the chicken house-keeping gene ggPRX

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In order to disclose the principles of gene assembly into transcription factories, we have studied spatial interactions of the chicken house-keeping gene ggPRX with other genes. Using the 4C procedure followed by parallel sequencing of ligation products, we have demonstrated that this gene preferentially interacted with all gene-rich regions in the same chromosome (chromosome 14) as well as with gene-rich regions in other chromosomes. The observed interaction patterns were very similar although not identical in cells of erythroid and lymphoid lineages. The major difference between erythroid and lymphoid cells was that in erythroid cells much less ligation products were mapped to the other chromosomes. This may reflect more compact organization of chromosomes in erythroid cells. Although the viewpoint (ggPRX gene) is located at a distance of 3 kb upstream to the alpha-globin gene cluster, no preferential interaction of this region with erythroid-specific genes was observed in chicken erythroleukemia cells. Few interacting regions specific for erythroid cells did not contain known erythroid genes and were not enriched in binding sites for erythroid-specific transcription factors. Based on these observations we suggest that association of genes into transcription factories occurs for the most part stochastically.

SW01.S1–51

Conformations of the mononucleosome in different ionic environment

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Eukaryotic genomic DNA exists as highly compacted nucleosome arrays called chromatin. DNA transcription, replication and repair are firmly connected with structural chromatin rearrangements. Numerous in vitro experiments have revealed that higher order chromatin rearrangements are realized through changes of interactions between the 30-nm fibers and reorganizations in the fibers, but detailed mechanisms of these transformations are not clear. It is likely that along with the influence of linker histones and other proteins the core histone octamer has also a leading role in these processes. As shown by the studies of nucleosomes in the fibers, the histone octamer can stably accommodate from 100 to 170 bp of DNA. So that the nucleosome core particle with 146 bp of DNA (1.75 turns) is no more than just one of possible stable variant in row of nucleosomes with different DNA length. Ionic environment is a permanent factor in the cell nucleus, providing native chromatin state. Lowering ionic strength leads to unwrapping of DNA from octamer, as well as to dissociation and unfolding of chromatin fibers, as it was shown in numerous studies in vitro and in situ. However, there is an obvious lack of information from mononucleosome level studies. What is the exact territory of the octamer on the DNA helix, if exclude other interactions occurring in chromatin array? And how salt conditions affect the interaction of the octamer and DNA?

Mononucleosomes were reconstituted from 353 bp DNA, containing strong nucleosome positioning sequence, and pure core histones with lack of posttranslational modifications. Atomic force microscopy images of the nucleosome were analyzed for wrapped DNA length. We have demonstrated that single core histone octamer may accommodate up to two full turns of DNA without being incorporated in fiber where cooperative internucleosome interactions are facilitated and more DNA wrapped around octamer may be stabilized. Moreover, we have shown the distribution of mononucleosome conformations in solutions with different ionic environment. The data have clarified that it is the presence of bivalent cations but not monovalent cations in solution supports the formation of mononucleosomes with longer DNA wrapped around the core histone octamer.

SW01.S1–52

Inorganic polyphosphate triggers interleukin 11 production in osteoblasts cell signalling

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Inorganic polyphosphates (polyP) are linear long-chain polymers that are known to be abundant in bone and cartilage yet their functions are little understood. The crosstalk between orthophosphate, pyrophosphate and polyphosphate remain unclear. We have been investigating the impact of high levels of polyphosphate on osteoblast cells and have been investigating the specificity of response compared to orthophosphate and pyrophosphate. Extracellular polyP increased proliferation rates, migration rates and rates of wound healing of human osteoblast-like SaOS-2 cells. Using microarrays we observed 29 genes upregulated and 18 genes downregulated specifically in response to long chain polyphosphate. Among them interleukin 11 (IL-11) and soluble carrier family 30 (zinc transporter) member 1 (SLC30A1) were the most upregulated and downregulated respectively. Further experiments validated the microarray results and we also observed that interleukin 11 was elevated at the protein level specifically in response to polyphosphate. A new model of interleukin 11 production in response to polyP is proposed as a mechanism of crosstalk between polyP and cytokine signalling.

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SW01.S1–53

Inhibition of nuclear actin polymerisation alters genome architecture in transcriptionally active avian and amphibian oocytes

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Avian and amphibian oocytes are highly transcriptionally active during certain period of diplotene in meiosis I. Intranuclear structures in amphibian and avian oocyte giant nucleus, or germinal vesicles, at the stage of transcriptional activity are scattered in a great volume of nucleoplasm, with decondensed lambrush chromosomes distributed chaotically in the central part of the nucleus and occupying the most of the nuclear volume. Proceeding to the sequential stages of oocyte growth is accompanied by condensation of chromatin, loss of transcriptional activity and approaching of nuclear structures in a small nuclear volume to form a karyosphere, which occurs before nuclear envelope breakdown. The mechanisms underlying dynamic changes of nuclear architecture during oocyte growth and karyosphere formation remain largely unknown. It was established, that amphibian germinal vesicles amass nuclear actin from the very beginning of oocyte development due to selective block of actin-exporting mechanism. The role of nuclear actin in maintenance of functional architecture of nuclear structures within the germinal vesicle was not studied so far. We found that actin enrichment in the oocyte nucleus is evolutionary conserved among amphibians and aves. Furthermore, we tested consequences of global inhibition of
actin polymerisation on nuclear architecture in giant nucleus of the growing oocyte in amphibian (clawed frog) and avian (chicken, quail and chiffinch) species. Exposure of oocytes to either cytochalasin D or latrunculin A induced dramatic changes in nuclear architecture, resembling those in later oocytes. Loss of chromosome positions in avian nuclei and aggregation of nuclear bodies and chromosomes in amphibian nuclei were observed after 2 h oocyte incubation with drugs. Prolonged drug treatment led to complete collapsing of nuclear structures in small nuclear volume. Stabilization of actin polymerisation by phalloidin applied to living oocytes did not alter spatial positioning of intranuclear structures, but triggered actin meshwork formation, which could not be detected in freshly isolated nuclei. Our data show that enrichment of actin in oocyte nuclei is essential for stabilizing the specific nuclear and genome architecture at the diplotene stage.

SW01.S1–54
The role of Drosophila chromatin remodeling factor CHD1 in replication-independent nucleosome assembly and in chromosome organization
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The organization of chromatin affects all aspects of nuclear DNA metabolism in eukaryotes. H3.3 is an evolutionarily conserved histone variant and a key substrate for replication-independent chromatin assembly. A thorough understanding of the replication-independent mechanisms of chromatin assembly, however, is lacking. The highly conserved chromatin remodeling factor CHD1, together with the chaperone NAP-1, assembles nucleosome arrays from DNA and histones in vitro. We investigated the role of CHD1 in chromatin assembly in vivo in Drosophila. CHD1 is implicated in genome-scale, replication-independent nucleosome assembly during reorganization of male pronucleus after fertilization. Elimination of CHD1 compromises incorporation of H3.3 into the male pronucleus and leads to the development of haploid embryos. At later stages of development deposition of H3.3 occurs in transcriptionally active chromatin and it is triggered by transcription. CHD1 has been implicated in transcription elongation-related chromatin remodeling and co-localizes with elongating form of Pol II. Due to the high material contribution of CHD1 gene products, the protein persists into third instar larval stages in Chd1 mutant larvae. To investigate the CHD1 functions, we generated a UAS-CHD1 (K559R) transgene producing a dominant-negative form of the CHD1. The over-expression of dominant-negative as well as native forms of CHD1 in salivary glands leads to appearance of decondensed polytene chromosomes with large additional puffs. In wild type flies CHD1 perfectly co-localizes in polytene chromosomes with large additional puffs. In wild type flies CHD1 perfectly co-localizes in polytene chromosomes with a truncated H3.3 protein (H3.3core-GFP) that can only participate in the replication-independent histone deposition. The H3.3 histone incorporation is compromised at decondensed regions of chromosomes in flies over-expressing CHD1. Expression of the H3.3 histone in salivary glands along with CHD1 under the control of the same GAL4 driver partially rescues the CHD1 over-expression phenotype, suggesting an existence of causal relationships between the effects of CHD1 over-expression on histone incorporation/exchange and on chromosome organization. Our findings suggest that CHD1 plays a major role in replication-independent nucleosome assembly and in chromosome organization in Drosophila.

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SW01.S1–55
Molecular modeling and SANS spectra simulations of alternative nucleosomal structures
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The organization of DNA into nucleosomes has profound implications for many key cellular processes such as differentiation. In order to understand how DNA accessibility is regulated, it is important to identify the driving force for nucleosome assembly and disassembly. These processes can not be fully understood without considering partially assembled nucleosomal structures, and how these may be affected by histone PTMs. In this work we made an attempt to construct models of several partially assembled nucleosomal structures that were proposed to occur on the nucleosome assembly pathway. These include the histone, tetrasome, and hexosome. The structures were constructed with the help of atomistic MD simulations in explicit and implicit solvent.

On the 100-ns timescale, the histone cores of three modeled structures mostly maintained their initial contacts with DNA and between protein subunits tightly fastened by four helix bundles. Partial unpeeling of the DNA off the histone core occurs in regions where it lost contacts with histones, removed from initial nucleosome structure. In hemiosome C-terminus tail of H2A, as well as N-terminus tails of H2B and H3 prevents unwrapping of DNA. In tetrasome this function is fulfilled by two N-terminus tails of H3. In hexosome all histone tails excluding two C-termini of H3, one of H4 and one of H2B participates in stabilization of coiled DNA structure. Unwrapping of DNA in tetrasome was achieved by temporally neutralizing of two H3 N-terminus tails upstream of residue 56, that mimics acetylation and methylation PTMs.

Obtained spatial structure of tetrasome was used as an elementary unit for construction of fractal supernucleosomal chromatin structure model, excluding self-intersection of individual nucleosome particles (J Phys Conf Ser 2012 351 012007). Unique algorithm was used to calculate SANS spectra of generated chromatin model (J Appl Phys 2011 110 102217). Comparison of SANS spectra simulated for fractal chromatin structure composed of tetrasomes with one composed of intact nucleosomes shows better agreement of the former with experimentally obtained SANS spectra of rat C6 glioma, HeLa and chicken erythrocytes nuclei, especially at high q.

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SW01.S1–56
The phenomenon of fractal organization of chromatin nuclei of eukaryotes by SANS
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The model of DNA packaging into chromatin of eukaryotic cells interphase nuclei, which was the matter of debates in recent years, suggests a fractal organization of the supernucleosomal
structure of the double helix. The results of confocal microscopy, small-angle neutron scattering and Hi-C techniques all suggest a two-phase fractal organization of chromatin and, in some cases, allow to build three-dimensional models of the genome. In this work we present the experimental data obtained by SANS on the nuclei of such cells as chicken erythrocytes, HeLa, glioma (C6) and drosophila embryos in which chromatin was fixed by glutar-aldehyde. SANS curves were obtained on KWS2 and KWS3 spectrometers, located on the research reactor FRM-II in Munich (Germany), in the range of the scattered vectors between 0.00029 and 0.25/A, which corresponds to a range of linear dimensions of a scatterer 2.5 nm–2.2 μm covering almost the entire hierarchy of the chromatin structure. Scattering measurement was carried out at two D2O content in H2O, 99% and 40%. Scattering curves obtained at 40% D2O characterize the structure of nucleic components of chromatin (DNA + RNA). For all the nuclei the form of these curves has a general qualitative feature: curves in the double-logarithmic coordinates have two linear regions, characteristic for the fractal structure, and the point of crossover. Quantitatively, the slope of the linear parts of the scattering curves to the right of the crossover point (large angles) was: chicken erythrocytes D_{H2O} = -2.2, HeLa = D_{H2O} = -2.25, C6 D_{C6} = -2.15 and drosophila embryos D_{DE} = -2.05 and left of the crossover points, respectively: D_{H2O} = -3.4, D_{H2O} = -3.96, D_{C6} = -2.8 and D_{DE} = -3.3. Crossover points: QC6 = 0.002/A (approximately 300 nm), QC6 = 0.004/A (approximately 150 nm), Q_{C6} = 0.009/A (70 nm) and Q_{DE} = 0.012/A (50 nm). Phenomenologically this mean that in all investigated nuclei nucleic acid component of chromatin structure on the scales below the crossover point has the properties of a mass fractal (close to a Gaussian coil), and the structure to the left of the crossover has the properties close to a fractal surface.

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**SW01.S1–57**

Bioinformatic and proteomic analysis of transcription factor binding sites in interacting regulatory elements in mouse T cells

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Genome organization, gene positioning and chromosomal interactions are key features in the highly compartmentalized and dynamic environment of the eukaryotic nucleus. Long range genomic interactions juxtaposing several regulatory elements have been previously described in mouse T cells using the Chromosome Conformation Capture (3C) method. The nature of these interactions in terms of protein factors that mediate and/or stabilize these interactions is unclear. Here, we present preliminary data using both in silico (motif finding algorithm for the interacting DNA sequences) and experimental (DNA affinity chromatography coupled to Mass Spectrometry) approaches to identify and characterize such protein factors in an unbiased way.

**SW01.S1–58**

Distribution of tandem repeats in human genome

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The objective of our work was to study characteristic distributions of tandem repeats (micro- and minisatellites) in the human genome. It is known that many microsatellites are associated with poly-A retroposon tails. We have observed that the sequence of the microsatellite repeated unit correlates with the length and subfamily of retroposon the microsatellite is associated with. Moreover, we have found that microsatellites with similar sequences of their repeated units tend to be located close to each other in the genome; this effect is visible for distances up to several thousands of base pairs, which makes it unlikely to be the repeat finder artifacts. Particularly dense clusters were observed for tandem repeats rich with frequent polymorphic positions. Clusters of such microsatellites are associated with regions of chromosomal structural variations and segmental duplications. We have suggested possible mechanisms explaining the observed phenomena.

**SW01.S1–59**

Neonatal cardiomyocyte excessive genome accumulation and HIF-1A overexpression after neonatal gastroenteritis: all or nothing response to disease

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Inflammation, growth retardation, malnutrition and cardiac overload during early childhood play an important role in the developmental programming of adult cardiovascular diseases. The common causes of these maladies in children are infectious gastroenteritis triggering malnutrition and tachyarrhythmia. Our recent follow up studies indicated that neonatal cryptosporidial gastroenteritis was associated with long-term cardiomyocyte abnormalities. The aim of the present study was to find out how neonatal cryptosporidiosis of various severities affects cardiac anatomy and cardiomyocyte remodeling. Using real-time PCR, cytometry, immunohistochemistry, image analysis and interatrial septum visual examination, we revealed that gradual increase in cryptosporidial invasion was associated with ‘all-or-nothing’ changes. At weak parasitic infection, interatrial septum was closed and there were no statistically significant changes in cardiomyocytes. At moderate and severe infections, all changes in cardiac anatomy and cardiomyocytes were statistically significant and demonstrated approximately similar degree. Compared to control, heart were atrophied and elongated, interatrial septum contined a small window (patent foramen ovale), cardiomyocytes lost protein, became thin and elongated and accumulated additional genomes. Also we found HIF-1α mRNA overexpression. Remarkable, the ‘all-or-nothing’ response to gradual stimulus strengthening is an important criterion of developmental programming, since such a response is commonly a consequence of abnormal anatomic structure formation cell differentiation failure. Our results could be interesting for physitians because they
indicate that moderate cryptosporidiosis can be dangerous for neonatal heart and can trigger cardiovascular pathology neonatal programming. Also our results for the first time demonstrate the association between gastroenteritis, patent foramen ovale and cardiomyocyte hyperpolypliodization.

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SW01.S1–60
Regions, associated with internal telomere repeats, in chromatin structuring
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Telomeres consist of guanine-rich repetitive sequences of nucleotides, which, together with specific proteins, form the telomeres and play an important role in the maintenance of eukaryotic genome integrity by protecting the ends of chromosomes, serving as substrate for chromosome end elongation by telomerase and thus preventing their replicative shortening. Telomeres also marking chromosome ends as allowed double strand breaks. Long terminal tracks of telomere repeats are associated with heterochromatin, and they regulate the epigenetic modifications, transcriptional activity and replication time of neighboring genome regions.

Although the functions of terminal telomere repeats are almost elucidated, there are also internal telomere repeats, the role of which remains unclear. In part, internal telomere repeats may be a reminiscence of some past Robertsonian-like fusions without telomere loss, but there are also a lot of relatively short internal telomere tracks, which are abundant and may play a role in genome structuring.

We supposed that some internal telomere repeats may be associated with regions with high frequency of specific breakage, and focused on the study of regions, flanked by inverted telomere repeats containing at least a single-stranded break on both sides, in the fish Danio rerio. We found that the average length and distribution of such regions is different between organs, and changes with age. Besides telomere repeats at ends, they also contain sequences, which are repeated in genome and partially do not correspond to known classes of repetitive elements. For some telomere-flanked regions the transcriptional activity was proved, and in the majority of them are predicted open reading frames, either corresponding to hypothetical proteins and peptides or homologous to known proteins. We also found, that the labeled probe to one of the telomere-flanked regions reveals particular chromatin structures in fibroblast nucleus, in the organization of which a major role plays the RNA. Thus the telomere-flanked regions may play a role in chromatin organization and genomic imprinting.

SW01.S1–61
Sequencing and comparative analysis of plastid genomes of non-photosynthetic plants
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Plastid genomes of non-photosynthetic plants represent an intriguing example of evolution in the apparent absence of functional constraint. The primary function of plastids is photosynthesis so the structure and evolution of plastid genomes in photosynthetic plants are extensively studied. In contrast little is known about the plastomes of non-photosynthetic species. In order to fill this gap we characterized plastid genomes in several lineages of non-photosynthetic plants and their photosynthetic relatives using next generation sequencing. We assembled complete or partial sequences of plastomes for six completely non-photosynthetic plants: Petrosavia stellaris, Monotropa uniflora, Neottia nidus-avis, Aphyllorchis pallida, Epipogium aphyllum and E. roseum and for Corallorhiza trifida and Epipactis helleana – the species which are partially mycoheterotrophic, but retain photosynthetic activity. As expected, in non-photosynthetic plants most genes related to photosynthesis, are lost or pseudogenized, and the size of the plastome is reduced. However, in contrast to previous observations, the degree of reduction and reorganization greatly differs presumably reflecting the time of transition to heterotrophic way of life. In P. stellaris rbcL and atp genes are conserved which indicates on rather recent transition. N. nidus-avis retains gene order typical for its photosynthetic relatives, while in other species plastomes are highly rearranged. In Epipogium aphyllum plastome reorganization and reduction is maximal: our estimate of its size is approximately 30 kb – the lowest found in angiosperms. Thus we demonstrate that despite the increased knowledge on plastid genomes an important modus of non-photosynthetic plastomes’ evolution, related to genome rearrangements, remained overlooked.

SW01.S1–62
Similar patterns of satellite DNA organization in mammal genomes
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Tandemly repeated DNA represents a significant portion of eukaryotic genomes. The large tandem repeats including satellite DNA are the main component of centromeric and pericentromeric regions that are mostly un assembled in the reference genomes. The incomplete characterization of large tandem repeats and satellite DNA limits experimental studies. We have recently published a bioinformatical approach to genome-wide large tandem repeats classification in mouse genome. Here, we present a comparative analysis of large tandem repeats from 20 mammal genome assemblies including ten primate genomes. Large tandem repeat families from different unrelated genomes exhibit similar sequence-independent patterns of distribution over GC-content, monomer length, and monomer variability. We found several similarly organized families that are briefly described below in order of decreasing size. The largest family (60–80% of all repeats) is formed by AT-rich (rodents and primates) or GC-rich (carnivores) repeats with complex high-order structure, long monomer, centromeric-pericentromeric chromosome position.
The largest family includes smaller centromeric subfamily characterized by presence of CENP-B box. In several cases centromeric CENP-B box positive family is formed by different tandem repeats (e.g. mouse minor satellite). The second largest family is formed by tandem repeats with relatively short monomer, large complex arrays and probably multi locus chromosome-specific variants (e.g. human classic satellites or mouse TR-21A-MM). Other smaller families with monomer size 20–40 bp are characterized by different from previous families GC-content, large complex arrays, and pericentromeric or subtelomeric multi locus location. A separated group of large tandem repeats is formed by complex arrays, and pericentromeric or subtelomeric multi locus localization. A separated group of large tandem repeats is formed by complex arrays, and pericentromeric or subtelomeric multi locus localization. Uncovering of common patterns of genome-level tandem repeats organization is the key to understanding tandem repeat functions and evolution.

SW01.S1–63
Methylation status of telomerase reverse transcriptase and telomerase RNA genes in Danio rerio at different ages

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Gene expression often depends on the methylation status of the regulatory region of the gene. DNA methylation may affect chromatin compaction and prevent the binding of transcription factors. We analyze the methylation status of CpG islands inside and near telomerase reverse transcriptase (TERT) and telomerase RNA (TR) genes in Danio rerio at different ages. We found that the regulatory region of the TERT gene (from −1500 to 0 bp) is methylated almost entirely, while the initial region of the gene (from 0 to 500 bp) is unmethylated. Besides, the methylation pattern does not depend neither on age nor the place in different organs. Similarly, the entire TR gene is unmethylated in all cases. It is interesting that the methylation status of the region right after the end of the TR gene depends on age. Thus, we believe that it is possible to regulate the transcription termination of this non-coding RNA using methylation.

SW01.S1–64
Mutations in ANTXR1 cause GAPO syndrome inherited disorders

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GAPO syndrome is a condition characterized by growth retardation, alopecia, pseudoanodontia, and progressive visual impairment. To identify the genetic defect in GAPO syndrome, we performed genomic analysis in four unrelated and ethnically diverse families. To localize the disease gene, we first genotyped genomic DNA from all three Czech family members using Affymetrix GeneChip Mapping 6.0 Arrays. Our analysis of the Czech proband did not reveal any rare and potentially disease-causing deletion or amplification larger than 10 kb, but we identified two extended autozygous regions on chromosome 2 and chromosome 4 containing 114 and 29 genes, respectively. To directly identify potential disease-causing mutations, we sequenced and analyzed the exomes of all three individuals from the Czech family.

Analysis of obtained data revealed in the proband 121 candidate variants. However, the only relevant variant compatible with a recessive disorder was a homozygous nonsense mutation (c.505C>T; p.Arg169*) in the ANTXR1 encoding anthrax toxin receptor 1 (ANTXR1).

In parallel, DNA samples from two other cases were independently analyzed using exome sequencing and we identified homozygous nonsense mutations (c.262C>T; p.Arg88*, c.505C>T; p.Arg169*) or splicing mutations (c.1435-12A>G; predicted to encode p.Gly479Phes*119). The nonsense mutations predictably trigger nonsense-mediated mRNA decay resulting in loss of ANTXR1. The splicing mutation theoretically encodes for a truncated ANTXR1 containing a neo-peptide on its C-terminus. The pathogenicity of mutations was studied on mRNA and protein levels in patients’ cultured fibroblasts, using RT-PCR, Western blot and immunofluorescence analysis.

The major phenotypic features of GAPO syndrome, which include dental abnormalities and accumulation of extracellular matrix, recapitulate those found in Anxtr1−/− mice and point towards an underlying defect in extracellular matrix regulation.

We conclude that our data, together with recapitulation of many of the phenotypic features characteristic of GAPO syndrome in Anxtr1−/− mutant mice strongly suggest causal involvement of ANTXR1 mutations in the generalized defect in extracellular matrix homeostasis characteristic of this disease.

SW01.S1–65
Spatial changes of HSA6, HSA12, HSA18 and HSAX centromeres in the interphase nucleus of MSC during cultivation and differentiation

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Positioning of chromosomes and their parts in the interphase nucleus is not random and seems to be an important mechanism of epigenetic regulation. Study of chromosome organisation is of particular interest in stem cells such as mesenchymal stem cells (MSC) because they are widely used in regenerative medicine and are of great value as a biological model capable of differentiation in different cell-lineages. The aim of the research was to identify the differences in the positions of chromosomes 6, 12, 18 and X in MSC. Cells in early (up to 4) and late (6–10) passages were analyzed as well as cells after differentiation in osteogenic and adipogenic directions. Over 4000 nuclei from 19 cell cultures were analyzed using FISH with centromere probes. Radial distances (RD) were measured for each chromosome centromere. At late passages the median RD values of the distal HSA6 homologue changed its position from 0.78 to 0.81 while centromere of HSAX in male cultures moved centrally (median values are 0.7 and 0.61 for early and late passages respectively). After adipogenic differentiation proximal homologues of both HSA18 and HSA12 moved to the periphery (median values are 0.34 and 0.39 for HSA18, 0.45 and 0.5 for HSA12 before and after differentiation respectively). In addition centromeres of HSAX showed statistically different position in male and female cultures which might be explained by different levels of activity of HSAX in male and female cells. The detected changes in the structure of the nucleus are new karyological characteristics of human MSC.
SW01.S1–66
Nuclear localization of beta2-tubulin in A431 cells
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There are increasing evidence that many types of cytoskeletal proteins are localized in nucleus. The fact of the nuclear localization of tubulin raises a lot of doubts. The goal of this study is to demonstrate the nuclear localization of tubulin in the isolated nuclei by confocal microscopy. This technique allows excluding the false-positive signal from cytoplasmic tubulin. It’s important to use method of subcellular fractionation with an evident efficacy in concerning of possible contamination of nuclear samples by cytoplasmic proteins. We have compared several widely used techniques. Analysis of the isolated nuclei by confocal microscopy has indicated that there are circles of actin filaments in the outside of their surfaces. Therefore, we have developed new method with better efficacy, which includes two-step purification of isolated nuclei. The purity and the integrity of the nuclei isolated were determined by fluorescent confocal microscopy. Analysis has indicated that there are no actin structures and microtubules on the surface of the isolated nuclei. The native structures of the nucleus are preserved. Thus, we have demonstrated the efficacy and the eligibility of this method for the subsequent analysis of the nuclear localization of tubulin. Using the confocal microscopy we showed that beta-2 tubulin is a part of the nucleoplasm of A431 cells whereas alpha-tubulin is not. These findings will be further used for studying the nuclear functions of tubulin.

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SW01.S1–67
The effect of linker histones on nucleosome distribution in Arabidopsis
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Linker histones are conserved and abundant structural components of eukaryotic chromatin that are thought to influence its compaction and hence, the accessibility of DNA for regulatory proteins. They have been shown to influence chromatin condensation, DNA repair, and nucleosome remodeling and have a role in directing the localization of epigenetic marks. Most of higher eukaryotes have multiple non-allelic forms of linker histones that are often highly redundant functionally, which makes the studies of their true biological role very difficult. Moreover, in animals the simultaneous knock-out of several major linker histone variants is embryo lethal. Arabidopsis, on the other hand, has only three linker histone variants (H1.1, H1.2 and H1.3) and remains viable even after almost complete knock out of all of them. This makes it a particularly good model to study the impact of these proteins on chromatin.

We will present a multi-level analysis of MNase treated nuclei from mutants in single, double and triple H1 genes, demonstrating their influence on nucleosome occupancy and chromatin structure in Arabidopsis.

SW01.S1–68
Function of Daxx/ATRX complex at centromeric and pericentromeric heterochromatin
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Nuclear structures ND10/PML NBs are linked to multiple processes, including the maintenance of intranuclear homeostasis by sequestering proteins into ‘nuclear depot’. This function presumes release of proteins from PML NBs and their redistribution to the alternative, supposedly ‘active’ locations, in response to the external stress application. To further investigate this nuclear depot function, we focused on the intranuclear distribution of proteins Daxx and ATRX that in normal conditions are mainly accumulated at PML NBs, and have a minor association with centromeres and pericentromeres (CEN/periCEN). Here we report that application of physiological Heat Shock (HS) changes this balance forcing very robust and reversible accumulation of Daxx on CEN/periCEN heterochromatin.

To understand functional consequences of Daxx deposition at CEN/periCEN, we tested for Daxx-dependency of heterochromatin transcription. Depletion of Daxx reduces accumulation of CEN RNA in normal conditions and periCEN RNA after HS application. Searching for the mechanism of Daxx-dependent regulation of heterochromatin transcription, we found that depletion of Daxx decreases incorporation of transcription-associated histone H3 variant, H3.3, into both CEN and periCEN. Surprisingly, HS-induced deposition of Daxx does not further elevate incorporation of H3.3 into CEN/periCEN that remained steady during stress and recovery. Instead, depletion of Daxx leads to HS-induced changes in the balance of epigenetic modifications at heterochromatin, most dramatically elevating levels of active H3K4Me2 modification at periCEN. We propose dualistic function of Daxx-containing complexes at CEN/periCEN: (i) regulation of H3.3 loading in normal conditions, when Daxx has minor association with these regions of genome, and (ii) protection of epigenetic status upon stress-induced robust accumulation, thus collectively guarding epigenetic identity of heterochromatin and genome integrity. Mechanism of Daxx/ATRX complex association with CEN/periCEN will be discussed.

SW01.S1–69
The breakpoint cluster regions of ETO gene involved in (8;21) leukemic translocations are enriched in acetylated histone H3
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One of the most frequent chromosomal translocation found in patients with acute myeloid leukemia (AML) is the t(8;21). This translocation involves the RUNX1 and ETO genes. The breakpoints regions (BCR) for t(8;21) are located at intron 5 and intron 1 of the RUNX1 and ETO gene respectively. To date, no homologous sequences have been found in these regions to explain their recombination. The BCR of RUNX1 gen is characterized by the presence of DNase1 hypersensitive sites and topoisomerase II cleavage sites, but no information exists about complementary region of ETO gene. Here we report analysis of
In human cells, the BCR at the ETO gene is enriched in hyperacetylated histone H3 compared to a control region of similar size where no translocations have been described. Moreover, acetylated H4 associates with both the whole ETO-BCR as well as the control intron. Interestingly, we observed no H1 association either at the BCR or the control region of the ETO gene. A common chromatin structure enriched in acetylated histones is present in breakpoint regions involved in formation (8;21) leukemic translocation.

**SW01.S1–70**

**Nuclear factor of activated T cells (NFAT) as a key control of endothelial cell phenotype**

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The mechanisms controlling the switch between pro-angiogenic and pro-inflammatory states of endothelial cells are still poorly understood. There is increasing evidence pointing at NFAT as a key transcription factor in endothelial cells. Herein we show the influence of NFAT in two different cellular physiological processes in Human Umbilical Vein Endothelial Cells (HUVEC): tube formation and both pro-angiogenic (PDGF-BB) and pro-inflammatory (IL-6) cytokine production.

Inhibition of NFAT is known to inhibit tube formation in vitro. In order to demonstrate that this effect is specific of NFAT and it’s not due to some unspecific effect of inhibitors, HUVEC were transfected by nucleasefication with siRNA against NFAT1 or NFAT2, control siRNA, pBJ5 empty vector and pBJ5 expressing NFAT2 and then seeded in Matrigel™. Pictures were taken at 4–24 h. Upon transfection of the HUVEC with pBJ5-NFAT2, the ability of the HUVEC to undergo tubular morphogenesis was enhanced, while incubation of HUVEC with siRNA-NFAT2 prevented cell spreading and resulted in inhibition of tube formation.

In order to study the effect of NFAT in cytokine production, HUVEC were cultured for 24 h with starving medium in the presence of TNF-α or VEGF-A alone or with NFAT inhibitors (cyclosporin-A and 11-R-Vivit), and NFAT inhibitors alone. Samples from culture medium were taken and the amount of IL-6 and PDGF-BB was quantified by ELISA. As expected, TNF-α significantly stimulated IL-6 production, and this production was inhibited by both NFAT inhibitors cyclosporin-A and 11R-Vivit (p < 0.5), suggesting that NFAT is actively stimulating this process. Interestingly, in the case of PDGF-BB production, the more specific NFAT inhibitor 11R-Vivit significantly increased its release in cells stimulated with TNF and VEGF but also in non-stimulated cells. This could indicate that in this case NFAT is modulating PDGF-BB expression by inhibiting its production, because inhibition of NFAT did not diminish but increased PDGF-BB release.

In conclusion, our data reinforce the idea of NFAT as a key modulator of the phenotype of HUVEC at different levels in response to both pro-inflammatory and pro-angiogenic environment.

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**SW01.S1–71**

**Cracking ‘the junk’ in genomes of two strains of mice BL6 versus CD1. Novel perspectives for The Human Genome Project**

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Cracking the Mayo Code was not trivial and started move forward only when Yuri Knorozov made a suggestion about nature of deciphermet. Knorozov proposed same phonetic sounds may be encoded by different hieroglyphs. To understand what is encoded by ‘junk’ is to accept similar principle of multi component status of genes and their products. It has been proposed a large portion of functional mammalian genomic sequences lies outside of protein-coding exons and can be only transcribed into long noncoding RNAs. Despite an enormous collaborative effort, including ENCODE project, to decipher the roles of long non-coding RNA in mammalian biology and their expression are not completely understood. Long noncoding RNAs (IncRNAs) regulate diverse processes; regulation of transcription and translation, retransposition and poorly understood function such as keeping and coordinating a memory to cellular responses to stress.

Current the most unclear still remains role of ncRNAs in response to environmental stresses; heat, cold, UV radiation and especially, hypoxia (low oxygen). It must be noted that the resistance to hypoxia plays a significant role in the development of many diseases, such as coronary heart disease, arrhythmia, heart failure, cancer, diabetes type II and many others. Recently we founded a substantial difference in expression levels of lncRNAs between two mice strains that were used to study an effect of hypoxia on the gene expression in cardiac muscle. We decided to use CD-1 and C57BL/6J mice strains as a model to system because of their significant physiological differences in response to acute hypoxia; C57BL is known to have high degree of resistance to hypoxia while CD1 is not. The analysis of expression arrays from Agilent revealed changes in the activity of a small number of genes in the C57BL/6J (about 100), whereas CD1 has changes in more than 1000 genes. Intriguingly, transcriptome profiling of CD 1 mice shown many energy related mitochondrial genes coding by nuclear genes then C57BL/6J did not show anything. Data on the expression of ncRNAs were opposite: C57BL/6J mice have significant changes in expression level twice more ncRNA compared to number of ncRNAs in CD1 mice. We also founded the difference between the individual genomic position and copies numbers by doing blast search of sequences of IncRNAs from CD-1 and C57BL/6J in available human genomes.

At the end of the day, we have a bunch of loci and genes, but none of them do all that much to raise the risk of heart disease, says Eric Topol, a cardiologist and director of the Scripps Translational Science Institute in San Diego, California. To date, most large-scale genome-wide association studies (GWAS) is failed to carry out to identify risk highly predictive of genetic markers for heart disease as well as arrhythmia. The ultimate goal of our study is to search novel association between common human diseases and ‘junk’ or none coding sequences in the human genome.

Taking advantage of this knowledge, it is possible to capture information of hundreds of novel genomic loci that influence various complex diseases in the past few years.
SW01.S2 RNA World (I-S2)

SW01.S2–1
Eukaryotic polyribosomes: formation, and structural and functional transformations
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The polyribosome is a group of ribosomes strung on an mRNA thread and translating it sequentially in one direction, from the 5' terminus to the 3' terminus of the polyribonucleotide chain. In various electron microscopy (EM) studies of eukaryotic cells, different configurations of polyribosomes have been reported, such as circles, zigzags, double rows, helices. In our experiments we initiated the process of de novo formation of polyribosomes using a long-term cell-free translation system based on wheat germ extract, and managed to observe a number of discrete stages in the course of functional polyribosome life. The polyribosomes at different stages of their life course were studied using the methodologies of sedimentation analysis, classic EM, cryo-electron microscopy and cryo-electron tomography, as well as the measuring of their protein-synthesizing activity. The results were as follows. (i) The first stage of the functional polyribosome life was the formation of predominantly circular polyribosomes, with the small ribosomal subunits directed inside the cycle. As a circular polyribosome was being further loaded with ribosomes and became longer, two sides of the cycle could be stuck together into a double-row-like polyribosome with still circular topology of the mRNA chain. (ii) In the course of further loading with ribosomes, the opening of mRNA cycles occurred and thus the transition to the linear topology passed. The linearization and the proceeding loading with ribosomes led to the organization of the polyribosomes into planar twofold helices, visualized as zigzags, with keeping the linear topology of their mRNA. (iii) The overloading of mRNA with ribosomes (at the level of 45 ± 15 nucleotides per ribosome) resulted in the abrupt compactization of the polyribosomes with the formation of a dense, highly ordered 3D helical structure. This conformational transition was accompanied by decaying of the protein-synthesizing activity.

In this work the main experiments were performed by Zh.A. Afonina, V.A. Shirokov and V.D. Vasilyev (Institute of Protein Research, Russian Academy of Sciences), in collaboration with B.P. Klaholz, A.G. Myasnikov and J.-F. Ménétret (Institute of Genetics and Molecular and Cellular Biology, Department of Integrated Structural Biology, Illkirch, France).

SW01.S2–2
Structural studies of the ribosome complexes
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The mechanism for decoding based on X-ray structures of bacterial 70S ribosome determined at 3.1–3.4 Å resolution and modeling cognate or near-cognate states of the decoding center has been investigated. We show that the 30S subunit undergoes an identical domain closure upon binding of either cognate or near-cognate tRNA. This conformational change of the 30S subunit forms a decoding center that constrains the mRNA in such a way that the first two nucleotides of the A codon are limited to form Watson-Crick base pairs. When a U-G or G-U mismatch, generally considered to form a Wobble base pair, is at the first or second codon-anticodon position, the decoding center forces this pair to adopt the geometry close to that of a canonical C-G pair. This by itself or together with distortions in the codon-anticodon mini-helix and the anticodon loop causes the near-cognate tRNA to dissociate from the ribosome. Our study provides structural insights into a universal principle of decoding on the ribosome.

The complete structure of the full 80S ribosome from Saccharomyces cerevisiae at a resolution of 3 Å have been determined. The model includes nearly all the rRNA sequences as well as all ribosomal proteins, with the single exception of protein L1. The eukaryotic 80S and bacterial 70S ribosome shares 34 common proteins and eukaryotic ribosome has additional 45 unique proteins and bacterial ribosome has 22 additional unique proteins. The majority of eukaryotic specific elements are located on the periphery of the conserved core thus broadening the surface of interactions between the two subunits through additional eukaryotic bridges. The molecular interactions creating these bridges together with their eukaryotic-specific components can now be described in details. Our crystals capture the ribosome in two different conformations which are believed to reflect intermediate states in course of mRNA and tRNA translocation. The structural comparison of these states, which differ by the degree of rotation of the small subunit and the swiveling of its head with respect to the large subunit, provides a detailed description of conformational rearrangements as well as coordinated movements of intersubunit bridges.

SW01.S2–3
Connecting RNA biology and metabolism
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RNA-binding proteins (RBPs) orchestrate virtually all aspects of RNA biology. I plan to speak about the identification of ‘all’ mRNA binding proteins (the ‘mRNA interactomes’) of mammalian cells and yeast using a new system-wide approach that we refer to as ‘Interactome capture’ (1,2). We have identified hundreds of new RBPs that are significantly enriched over negative controls shown by analysis of independent repeat experiments. The described method is broadly applicable to study mRNA interactome composition and dynamics in varied biological settings. These data and unpublished work from hepatocytic Huh-7 cells and yeast shed new light on diverse aspects of RNA biology, including RBPs in disease and novel RNA-binding architectures (3). We also identify enzymes of intermediary metabolism that moonlight as RBPs in vivo, implicating these in the recently proposed REM (RNA/enzyme/metabolite) networks for the coordination of cell metabolism and gene expression (4).

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SW01.S2–4
Genome-wide ribosome profiling to define in vivo functions for Dom34, Hbs1 and Rli1
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There are several mRNA surveillance pathways in eukaryotes that moderate the effects of natural errors in the cell and more broadly regulate gene expression. We have recently defined bio-
chemical parameters of the no-go decay quality control system using our previously developed in vitro reconstituted yeast translation system. In these studies, we showed that two factors implicated in no-go decay (NGD) and non-stop decay (NSD), Dom34 and Hbs1, promote subunit dissociation and peptidyl-tRNA drop-off on the ribosome to initiate recycling events that eventually lead to mRNA decay. Of particular interest is that these factors, Dom34 and Hbs1, exhibit a biochemical preference for 3′ untranslated mRNAs, suggesting a biochemical outcome for a likely in vivo consequence of stalling during translation (endonucleolytic cleavage of the mRNA). Current efforts focus on a broad analysis of the in vivo targets of mRNA surveillance (including NGD, NSD and NMD) using a series of reporter constructs and genome-wide ribosome profiling.

**SW01.S2–5**

Modification of bacterial ribosome

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Modification of ribosomal components, rRNA and r-proteins are ubiquitous among living organisms yet its functional role is largely obscure. In our work we identified bacterial RNA methyltransferases responsible for the formation of m²A37 of tRNA²⁰¹, m⁶G966 of the 16S rRNA, m²A1618, m⁶G1835, m²A2030 and m²G2445 of the 23S rRNA. With identification of YhiR coding for the 23S rRNA m²A2030 methyltransferase in year 2012 the list of E. coli rRNA methyltransferases was completed.

The remaining problem is recognition of the modified nucleotides functional role. While involvement of modified nucleotides in the assembly of ribosomes is widely recognized, their role in regulation of translation is only in the beginning of the study. We demonstrated that nucleotides m⁶G966/m⁵C967 of the 16S rRNA, m²A1618, m⁶G1835, m²A2030 and m²G2445 of the 23S rRNA. With identification of YhiR coding for the 23S rRNA m²A2030 methyltransferase in year 2012 the list of E. coli rRNA methyltransferases was completed.

**SW01.S2–6**

Translation-dependent mRNA Decay

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Nonsense-mediated mRNA decay (NMD)¹ and the mechanistically related Staufen1 (STAU1)-mediated mRNA decay (SMD)² are critical pathways of cellular post-transcriptional control. NMD generally occurs when translation terminates upstream of an exon-junction complex, and it functions largely as a quality-control pathway that targets newly synthesized mRNA. In contrast, SMD occurs when translation terminates upstream of a STAU1-binding site (SBS), and it regulates primarily steady-state mRNA. New insights into the how the ATP-dependent RNA helicase UPF1, which functions in both pathways, associates with NMD substrates will be provided. Additionally, the roles of STAU1 dimerization, the STAU1 paralog STAU2 and long-noncoding RNAs, which we call ½-sbsRNAs, in the SMD of human and mouse cells will be discussed. Our studies of SMD in mouse define new functions for IncRNAs and B and identifier (ID) small interspersed elements (SINEs) in myogenesis that undoubtedly also influence many other developmental and homeostatic pathways.

**References**


**SW01.S2–7**

Telomerase biogenesis and regulation

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Telomerase is the ribonucleoprotein complex that replenishes DNA sequences at the ends of chromosomes using part of an RNA subunit as template for telomeric DNA repeat synthesis. Whereas insufficient telomerase activity is associated with various degenerative disorders including dyskeratosis congenital and aplastic anemia, high levels of telomerase activity are commonly found in cancer cells. These observations provide substantial impetus for studying the biogenesis pathway of this ribonucleoprotein (RNP) complex and the factors that modulate its activity. We will describe the sequence of events that lead from the transcription of the core telomerase subunits to the assembly of an active RNP. Our work provides insights into multiple layers of regulation that fine tune the amount of telomerase activity under different growth conditions.

**SW01.S2–8**

Biosynthesis and function of long non coding RNAs in muscle differentiation

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High throughput transcriptome analysis of the last years has revealed that the mammalian genome is pervasively transcribed...
into many different complex families of RNA. It is now becoming largely accepted that the non-coding portion of the genome rather than its coding counterpart is likely to account for the greater complexity of higher eukaryotes. In addition to a large number of alternative transcriptional start sites, termination and splicing patterns, a complex collection of new intronic, intergenic and antisense transcripts was found.

Although many studies have helped unveiling the function of many small non-coding RNAs, very little is known about the long non-coding (lncRNA) counterpart of the transcriptome.

By studying in vitro and in vivo muscle differentiation, we have identified several regulatory circuitries, required for proper muscle tissue development and function, which are controlled by small and long non-coding RNAs. The analysis of specific regulatory circuitries controlled by such mRNAs allowed to identify and to explain some pathogenetic traits of neuromuscular disorders.

The muscle-specific long non-coding RNA linc-MD1, was previously shown to be expressed in a short window of time during early phases of muscle differentiation and to trigger the switch to later stages. Linc-MD1 acts, through competition for miRNA binding, by activating mRNAs which share common miRNA sites.

Interesting features related to its biogenesis and mode of action will be presented.

**SW01.S2–9**

Antiviral RNA interference in mammalian cells

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While it is well established in plants and invertebrates, the role of RNAi in mammalian antiviral defence has been the subject of intense debate that is still unsettled. Here, I will show, in undifferentiated mammalian cells, how processive Dicer activity generates abundant small RNA populations from double-stranded (ds) RNA replication intermediates of two unrelated viruses. These Dicer-dependent small RNA populations form perfectly complementary duplexes with 2-nt 3′ overhangs, are phased in a 22-nt periodicity, and are loaded into mammalian Ago2 indicating, therefore, that they are bona fide virus-derived siRNAs. Interestingly, the process of cellular differentiation reduces, but does not alleviate viral siRNA production, providing a first possible explanation as to why it has been difficult so far to detect antiviral RNAi in mammalian cells. A second reason, which we anticipated from our previous work in plants, is that some mammalian-infecting viruses produce potent suppressors of RNAi (VSRs) that conceal siRNA production/interaction; this phenomenon will be dramatically illustrated in the context of one of the above-mentioned infections. I will finally show how the loss-of-virulence of a VSR-deficient virus is rescued in RNAi-deficient mammalian cells in the same way as was previously demonstrated in virus-infected plants or *Drosophila*. These results provide unequivocal evidence that antiviral RNAi operates in mammalian cells and will be further discussed in the context of long-established facts pertaining to long-dsRNA-triggered RNAi in mammalian cells and likely relevant to infections of adult organisms.

**SW01.S2–10**

Regulation of microRNA biogenesis and function

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MicroRNAs (miRNAs) guide Argonaute (Ago) proteins to target mRNAs leading to gene silencing. Some but not all Ago proteins can cleave the complementary target RNA. In human, only Ago2 is catalytically active while Ago1, 3 and 4 are inactive. We have mutated human Ago1 and Ago3 and generated catalytic Argonaute proteins. We find that two short sequence elements at the N-terminus are required for activity. In addition, Ago1 PIWI domain mutations may mis-arrange the catalytic center.

Ago proteins are not the actual mediators of gene silencing but interact with a member of the GW182 protein family (also known as GW proteins), which coordinates all downstream steps in gene silencing. GW proteins contain an N-terminal Ago-binding domain that is characterized by multiple GW repeats. Within the Ago-binding domain, Trps mediate the direct interaction with the Ago protein. In addition to the analysis of cleavage activity mentioned above, we have characterized the interaction of Ago proteins with GW proteins in molecular detail using biochemical and biophysical methods. We find that only some specific Trps engage in Ago interactions and these residues reside in fully unstructured protein environments. Using cross-linking followed by mass spectrometry, we map the GW protein binding site on Ago2 allowing for structural modeling of Ago-GW182 interactions. Based on our data, we provide a model in which the optimal spacing between two Trps defines high affinity Ago binding sites on GW182 proteins.

**SW01.S2–11**

Small RNA-based adaptive immunity of prokaryotes

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CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated genes) is a small RNA-based adaptive prokaryotic immunity system that functions by acquisition of short fragments of DNA (mainly from foreign invaders such as viruses and plasmids) and subsequent destruction of DNA with sequences matching acquired fragments. Some mutations in foreign DNA that affect the match prevent CRISPR/Cas defensive function. Matching sequences that are no longer able to elicit defense, still guide the CRISPR/Cas acquisition machinery to foreign DNA, thus making the spacer acquisition process adaptive and leading to restoration of CRISPR/Cas-mediated protection. Evidence will be presented suggesting that after initial recognition of partially matching foreign DNA, the CRISPR/Cas acquisition machinery moves along the DNA molecule, occasionally selecting fragments to be incorporated into the CRISPR locus. Our results explain how adaptive CRISPR/Cas immunity becomes specifically directed towards foreign DNA, allowing bacteria to efficiently counter individual viral mutants that avoid CRISPR/Cas defense.

**SW01.S2–12**

Preventing of translation stalling on consecutive proline codons by the translation factor EF-P

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EF-P is an evolutionary conserved translation factor that binds at the interface between the ribosomal subunits and mimics a tRNA bound between the P and E site. The function of EF-P and of its eukaryotic ortholog is called initiation factor eIF5A has long been unclear. We show that EF-P specifically catalyzes
translation of proline clusters such as PPP, PPG, PPPP, PPGG, and longer proline runs, which in the absence of EF-P cause ribosome stalling and lead to the synthesis of abortive peptides. Notably, many proteins that have poly-proline stretches and are likely targets of EF-P are key player in bacterial viability and virulence, including proteins of the flagellum, many metabolic enzymes and membrane transporters, and even proteins playing a key role during infection of eukaryotic host by enterohemorrhagic E. coli (EHPEC and EPEC). In the cell, EF-P is post-translationally modified by a hydroxylated beta-lysine residue attached to Lys34; we show that the modification increases the catalytic proficiency of the factor by 100-fold. EF-P appears to be an elongation factor which works only on subset of cellular proteins, which explains the highly pleiotropic effects of deletions of the genes coding for EF-P or its modification enzymes, but is fundamentally different from other translation factors.

**SW01.S2–13**

**tRNA-assisted editing mechanism in translation quality control**

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Aminoacyl-tRNA synthetases (aaRSs) maintain fidelity during protein synthesis by attaching amino acids to their cognate tRNAs. However, the affinity difference is not enough for the synthetases to discriminate strictly between similar amino acids. When a tRNA is acylated with the wrong amino acid this would lead to error in the incorporation of genetically coded amino acids in protein. To overcome this problem, several aaRSs have developed the ability to hydrolyze the mischarged tRNA in an extra editing domain. In this work we have studied molecular mechanisms of editing by synthetases from two different classes: *Thermus thermophilis* leucyl-tRNA synthetase (LeuRSSTT) from class I and *Enterococcus fecalis* prolyl-tRNA synthetase (ProRSEF) from class II. To understand the mechanisms of editing reaction for enzymes with absolutely different architecture of editing domains (1, 2), we have used a number of approaches, including molecular modeling, quantum-mechanical calculations, site-directed mutagenesis and enzyme modification of tRNA. Our intensive alanine scanning mutagenesis of LeuRSSTT and ProRSEF editing sites has failed to identify catalytic residues for hydrolysis within the active site. On the other hand, modification of tRNAPro at the 3′-OH of A76 and tRNALeu at the 3′-OH of A76 by replacing them each with a proton or fluorine, revealed an essential function for these groups in hydrolysis. On the basis of obtained experimental results and our QM/MM calculations we suggest a tRNA-assisted mechanism of post-transfer editing by LeuRS and ProRS in which 2′- or 3′-OH group of the substrate plays a key role.

**References**


**SW01.S2–14**

**Structure and function of the spliceosome**

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The spliceosome is a multi-MDa RNP machine that consists of the small nuclear (sn)RNPs U1, U2, U4/U6 and U5, and numerous non-snRNPs proteins. The stepwise interaction of the snRNPs with the pre-mRNA during spliceosome assembly culminates with the formation of the so-called B complex which still lacks an active site. During the subsequent catalytic activation step major RNA-RNA and RNP remodelling events occur, generating the activated B complex, which then catalyzes the first step of splicing to yield the C complex. We recently established an *in vitro* splicing complementation system that allows us to reconstitute both steps of yeast splicing with purified components and have now extended it to the disassembly stage of the spliceosome. Using this system, we have investigated the factor requirements and kinetics of the various remodelling steps of the yeast using fluorescence cross-correlation spectroscopy. We are also employing electron cryomicroscopy for the investigation of the 3D structure of yeast spliceosomes at defined stages of assembly. Finally, I will report on the crystal structure of two proteins involved in the catalytic activation of the spliceosome.

**SW01.S2–15**

**Recoding UGA as selenocysteine: idiosyncratic and shared factors for mRNP assembly and translation**

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Under certain circumstances, stop codons can switch from nonsense to sense. This is exemplified by UGA that, among other meanings, can encode the selenium-containing amino acid selenocysteine (Sec) in selenoproteins. These proteins, carrying Sec in the active site, fulfill as varied functions as defence against reactive oxygen species, sperm and thyroid hormone maturation, muscle biogenesis, etc. Reading selenocysteine instead of stop at the UGA codon is a complex event that requires both dedicated RNAs and proteins but also general factors. Mutations in some of these components or in the selenoproteins themselves may lead to embryonic lethality or various pathologies, attesting the importance of selenoproteins in health and disease. At the mechanistic level, the central question is to understand how assembly of a selenoprotein mRNA-protein complex occurs in an orderly and temporal fashion so that approaching ribosomes are cleared at the UGA Sec codon. Little is known regarding the issue. Recent findings will be presented that deepen our understanding of where and when selenoprotein mRNAs and the selenoprotein mRNA-binding protein SBP2 interact with the ribosome in the course of translation.

**SW01.S2–16**

**Nuclear non-coding RNA regulation**

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We have recently demonstrated that Mpn1 is an evolutionary conserved RNA exonuclease that trims the 3′-end oligouridine
SW01.S2–17
Diverse mechanisms of mRNA binding to ribosomes in mammalian cells
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The main focus of our current studies is mechanism(s) of mRNA recruitment to mammalian ribosomes. We focus on two aspects of this recruitment: large variations in the eIF4E- and cap-dependence of mRNA binding to 40S ribosomes and the problem of cap-independent translation of cellular mRNAs. Both points are very important for understanding of cancer progression and mechanisms of cell survival after drug treatment. The widely adopted hypothesis says that mRNAs with highly structured 5′ UTRs should require more helicase eIF4A and, hence, be more dependent on the cap and eIF4E. As most of oncogenic factors are encoded by such mRNAs, this hypothesis explains why many tumors have elevated concentrations of eIF4E.

Comparison of mRNA translation efficiency directed by different capped and uncapped 5′ UTRs in RNA-transfected cells did not support this idea [1]. There is no correlation between the length and degree of the secondary structure of a 5′ UTR and its cap requirement. These data are strongly supported by recent ribosome profiling experiments performed on mTOR-inhibited cells (2, 3). Particularly, the most cap-independent mRNA encodes apoptosis scaffold protein Apaf-1, though its 5′ UTR highly structured. Ironically, we did not find any IRES-element within highly structured 5′ UTRs of the Apaf-1 or other mRNAs with a low cap-dependence (1). Moreover, the Apaf-1 5′ UTR continues to use a rather effective scanning mechanism during apoptosis when eIF4E is sequestered by 4E-BP and eIF4G is partially cleaved (4). These data allow us to think that some cellular mRNAs are capable of cap- and IRES-independent translation initiation provided by Cap-Independent Translation Enhancers (CITEs). They may function in a way similar to CITE-elements found in uncapped mRNAs of some plant viruses. The proposed mechanism was supported by inserting an eIF4G-binding element from a viral IRES into 5′ UTRs of strongly cap-dependent mRNAs. Such insertion dramatically reduced their requirement for the 5′-terminal m7G-cap, though the translation remained dependent on a vacant 5′-end and occurred by a scanning mechanism (5). These data may substantiate a new paradigm of translational control under stress conditions.

References

SW01.S2–18
Characterization of factors regulating gene expression through mRNA decay and RNA quality control
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mRNA decay is a complex process that encompasses both the degradation of normal mRNAs after their translation, and the elimination of defective transcripts through Quality Control mechanisms. In the first case, mRNAs are usually deadenylated before being either decapped and exonucleolytically degraded in the 5′–3′ direction, or trimmed by the exosome. Quality Control mechanisms initiate with the recognition of defective mRNAs that are quickly degraded preventing the production of aberrant or defective proteins. In all cases, mRNA decay mechanisms are tightly associated with connected to mRNA translation.

Understanding mRNA decay requires deciphering the activity of factors involved in this process. In many cases, those are protein complexes whose organization must also be elucidated. Using as examples the CCR4-NOT complex involved in deadenylation, decapping factors and the Dom34-Hbs1 assembly involved in RNA Quality Control, I will present some of our recent results on the characterization of the composition, organization and function mRNA decay factors.

SW01.S2–19
Proteins forming the hepatitis C IRES binding site on the human 40S ribosome
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Initiation of translation of hepatitis C virus (HCV) genomic RNA is provided by a highly structured specific fragment in its 5′-untranslated region, the so-called Internal Ribosome Entry Site (IRES), which binds to the 40S ribosomal subunit without assistance of initiation factors, stimulating subsequent formation of the 48S pre-initiator complex. To identify ribosomal proteins (RPs) of the 40S subunit forming the HCV IRES binding site, we have applied a cross-linking approach utilizing HCV IRES derivatives bearing photocleavable groups at the nucleotides in the
SW01.S2–20
Non-coding transcripts of tandem repeats involved into formation of nuclear domains in growing oocytes
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Transcriptome in a cell consists of both protein coding sequences and a variety of non-coding transcripts, many of which have unknown functions. Here we present data on the transcriptional activity of novel tandemly repetitive DNA, subcellular localization of the resulting non-coding transcripts and RNP-composition of domains comprising such RNA in growing oocytes of birds. Using a combination of bioinformatic analysis of chicken genome and a high resolution FISH-mapping of chicken genomic DNA fragments on giant lampbrush chromosomes we identified a new non-coding tandem repeat that forms a cluster on the long arm of chicken chromosome 2. The identified tandem repeat is transcribed during oogenesis, transcripts taking part in the formation of nuclear structures that have unusual fibrillar morphol- ogy as determined by atomic force microscopy (so-called ‘lumpy loops’). The tandem repeat responsible for formation of complex loops on chicken lampbrush chromosome 2 was thus called ‘lumpy loop 2 repeat’ (LL2R). RNP-matrix of the ‘lumpy loops’ and their derivatives in the nucleoplasm accumulate large amounts of non-coding transcripts of LL2R repeat. In contrast to earlier described nuclear stress bodies, ‘lumpy loops’ that accumulate LL2R non-coding RNA attract splicing factor SR-protein SC35 and TMG-capped small nuclear RNAs leading to formation of nuclear structures enriched with spliceosomal components. At the same time, avian oocyte nuclei lack extrachromosomal interchromatin granule clusters. We assume that stable nascent transcripts of LL2R repeat associated with transcription unit on chromosome 2 are involved into formation of SC35-domains in chicken oocyte nucleus in a way analogous to Nuclear Enriched Abundant Transcript 1—a long non-coding RNA accumulated in paraspeckles in mammalian species. The complex loops with a high concentration of splicing factors and noncoding RNA on avian lampbrush chromosomes extend the list of nuclear domains that accumulate specific sets of RNP complexes at certain chromosomal loci and can be visualized by 3D microscopy. It is evident that formation of such depot in oocyte nucleus is nucleated by active transcription of non-coding tandem repeats, which strongly supports a model of RNA-mediated nuclear domain formation.

SW01.S2–21
Engineered ‘restriction RNases’ for sequence-specific cleavage of dsRNA and RNA in DNA-RNA hybrids
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Ribonucleases (RNases) are valuable tools applied in the analysis of RNA sequence, structure and function. Their substrate specificity is limited to recognition of single bases or distinct secondary structures in the substrate. Thus far, there have been no RNases available for purely sequence-dependent fragmentation of RNA, analogous to restriction enzymes for DNA. We have therefore searched for existing RNases that could be engineered to become sequence-specific. Using a combination of bioinformatics methods and experimental protein engineering we have obtained prototypes of two sequence-specific ‘restriction RNases’ (RRNases): one that cleaves both strands of dsRNA within a target sequence, and one that cleaves RNA within DNA-RNA hybrids at a particular distance from the target sequence.

Based on structural analysis of enzymes from the RNase HI superfamily we identified loops that could be extended to make specific contacts with bases in the dsRNA substrate. Biochemical characterization of selected members that possess extended versions of such loops revealed that some of them indeed exhibit sequence specificity. For one of such enzymes we constructed a structural model of a protein-RNA complex, and used it to guide site-directed mutagenesis aimed at elucidating the molecular basis of specificity and to increase the selectivity of cleavage. The obtained prototype RRNase recognizes a partially degenerated hexanucleotide target sequence and is capable of cleaving individual sites in long dsRNA molecules.

A prototype RRNase that cleaves the RNA strand in DNA-RNA hybrids five nucleotides from a nonanucleotide recognition sequence was constructed by fusing two functionally distinct domains: a non-specific RNase HI and a zinc finger that recognizes a sequence in DNA-RNA hybrids. The optimization of the fusion enzyme’s specificity was guided by a structural model of the protein-substrate complex and involved a number of steps, including site-directed mutagenesis of the RNase moiety and optimization of the interdomain linker length.

For both types of RRNases we implemented methods of specificity engineering, to enable generation of variants specific for other target sequences, making it feasible to acquire a library of enzymes that recognize and cleave a variety of sequences, much like the commercially available assortment of restriction enzymes. Potentially, RRNases may be used in vitro for production of RNA molecules with defined length and termini, which may be a cheaper alternative to chemical synthesis; they may be also used in vivo for targeted RNA degradation.
The long noncoding RNA TERRA and the telomeric DNA damage response

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Telomeres are the nucleoprotein caps that protect the ends of linear chromosomes from being recognized as sites of DNA damage. Telomeres elicit a DNA damage response upon shortening and loss of the telomeric shelterin component TRF2, leading to cellular senescence. We now identify roles of the long non-coding RNA TERRA and the lysine-specific histone demethylase LSD1 in the regulation of the MRE11 nuclease during the telomeric DNA damage response. Our data suggest a model in which TERRA promotes the recruitment of LSD1 to unapped telomeres by stabilizing the LSD1-MRE11 interaction. LSD1 then demethylates MRE11, stimulating its catalytic activity for nucleolytic processing of unapped telomeres.

The architecture of Tetrahymena telomerase holoenzyme

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Telomerase adds telomeric repeats to chromosome ends using an internal RNA template and specialized telomerase reverse transcriptase (TERT), thereby maintaining genome integrity. Little is known about the physical relationships among protein and RNA subunits within a biologically functional holoenzyme. We have determined the architecture of Tetrahymena thermophila telomerase holoenzyme by electron microscopy. Six of the seven proteins and the TERT-binding regions of telomerase RNA (TER) have been localized by affinity labeling. Fitting with high-resolution structures reveals the organization of TERT, TER, and p65 in the RNP catalytic core. p50 has an unanticipated role as a hub between the RNP catalytic core, p75-p19-p45 subcomplex, and the DNA-binding Teb1. A complete in vitro holoenzyme reconstitution assigns function to these interactions in processive telomeric repeat synthesis. These studies provide the first view of the extensive network of subunit associations necessary for telomerase holoenzyme assembly and physiological function.

SW01.S2–24
MicroRNAs and other regulatory RNAs

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We have been using molecular and computational approaches to find regulatory RNAs, identify the messages that they regulate, and then investigate molecular consequences of these regulatory interactions and their functions during development, oncogenesis, and other biological processes. This talk will describe our current understanding of microRNAs and other regulatory RNAs. Potential topics include how the cell distinguishes microRNA precursor transcripts from all the other transcribed RNAs, how microRNAs recognize their targets, the identities of the regulatory targets, the extent to which microRNA-directed repression can be explained by deadenylation or destabilization of the targeted mRNAs, and the genomics, evolution and functions of longer non-coding RNAs.

SW01.S2–25
Reversible RNA methylation in biological regulation

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Reversible methylation on DNA and histones is known to play critical roles in epigenetic regulation of gene expression. Mammalian messenger RNAs (mRNA) and non-coding RNAs are ubiquitously methylated on selected internal sites. We have recently shown that the human fat mass and obesity-associated protein FTO is the first RNA demethylase that can oxidatively remove the m6A methylation of mRNA and non-coding RNA. We have also found that a homologue of FTO, ALKBH5, is the second mammalian RNA demethylase that affects fertility. I will present our recent results on the reversible RNA methylation and the impact on biological regulation which appears to affect many aspects of RNA metabolism.
RNA function depends on its structure, yet three-dimensional structures of most biologically important RNAs are unknown. Many RNAs contain both highly structured and also functionally important flexible elements, limiting high-resolution structure determination using either X-ray crystallography or NMR. Therefore, it is important to computationally model RNA structures as an alternative. The vast conformational space makes the direct computational modeling of RNA folding difficult. We develop a multiscale approach for RNA folding using discrete molecular dynamics (DMD). DMD simulations feature rapid sampling of conformational space, and we use a coarse-grained representation to effectively model RNA structures. Benchmark study suggests that the DMD-based RNA model is able to accurately fold small RNA molecules. However, the large conformational space and force field inaccuracies make it difficult to computationally identify the native states of large RNA molecules. We devised an automated modeling approach for determination of large and complex RNA structures using experimentally derived structural information. We tested the structure determination method on several RNA molecules with known experimental structures. In all cases, we were able to find native states of these RNA molecules. Therefore, a combination of experimental and computational approaches has the potential to yield native-like models for the diverse universe of functionally important RNAs, whose structures cannot be characterized by conventional structural methods.

Mitochondria are essential organelles of eukaryotic cells, taking part in several critical cellular processes. They contain their own genome (mtDNA) packaged into supramolecular nucleoprotein complexes. Mutations in mtDNA have been associated with a wide variety of human disorders. In patients with mtDNA defects, it is common to find mutant and normal (wild type) mtDNA molecules in the same cell, a phenomenon known as heteroplasmy. Manifestation of biochemical and clinical defects occurs only when a threshold level of heteroplasmy (>60% of mutant genomes) has been reached. Since there is no effective treatment for these disorders, one attractive approach would be to specifically target mutant mtDNA to prevent it from replicating, thereby allowing propagation of wild type genomes.

This strategy is confronted to two problems: translocation of the anti-genomic oligomers through the double mitochondrial membrane, and their access and specific binding to mutated region of mtDNA. Our study of the natural pathway of RNA import into mitochondria permitted to identify the import determinants in tRNA and SS rRNA structures. Basing on these data, a set of small RNA molecules with significantly improved efficiency of mitochondrial import was constructed [Kolesnikova et al. (2010) RNA; Smirnov et al. (2011) Genes and Dev.]. To create a vector system able to target therapeutic oligonucleotides into deficient human mitochondria, we inserted into these RNAs short sequences corresponding to the boundaries of a large deletion in mtDNA associated with a neuromuscular syndrome KSS. Recombinant RNAs, introduced into cultured human cells containing KSS deletion, were shown to be stable in the cytosol, partially imported into mitochondria, and induced a decrease of the mutant mtDNA proportion, thus validating the potential of our approach to rescue the deleterious mtDNA mutations [Comte et al. (2012) Nucl. Acid Res.].

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Despite intensive studies concerning the structure and the transcriptional regulation of the three RNA polymerases, little is known about the mechanisms governing its assembly, transport to the nucleus, stability, degradation, and recycling. Our data help clarify the mechanisms governing biogenesis of the three eukaryotic RNA pols. We show evidence that Bud27 is the first example of a protein that participates in the biogenesis of the three eukaryotic RNA polymerases and the first example of a protein modulating their assembly instead of their nuclear transport. In addition, we demonstrate that correct assembly of RNA pol II is a prerequisite to properly maintain several transcription steps. In fact, assembly defects alter transcriptional activity, the amount of enzyme associated with the genes and its distribution, increases the amount of stalled RNA pol II at the promoter-proximal regions, affects the CTD phosphorylation, and interferes with mRNA capping machinery. In addition, our data show that TBP occupancy does not correlate with RNA pol II occupancy or transcriptional activity, suggesting a functional relationship between assembly, Mediator and PIC stability.

Dbp5 is a DEAD-box RNA helicase essential for mRNA export from the nucleus. Recent study has revealed a novel function for yeast Dbp5 in translation termination. But mechanisms of protein synthesis in yeast and high eukaryotes could be different. We cloned human homolog of Dbp5 (Ddx19) and produced recombinant protein in E. coli. Helicase activity of recombinant human Dbp5 was demonstrated using luciferase based assay. By gel filtration we have shown an ability of human Dbp5 to bind eRF1 in the presence and absence of ATP in vitro, but only 20% of both proteins assembled into complex. We suppose that for effective Dbp5-eRF1 complex formation additional factors are required. Activity of human Dbp5 in translation termination was tested in reconstituted in vitro eukaryotic translation system. We have shown appearance of the additional toe-print peak during termination. Probably binding of eRF1 with Dbp5 during translation changes conformation of ribosomal complex and regulates peptide release.

Mitochondrial diseases: modeling anti-genomic therapy by imported RNA
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Role of human Dbp5 in translation termination
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Dbp5 is a DEAD-box RNA helicase essential for mRNA export from the nucleus. Recent study has revealed a novel function for yeast Dbp5 in translation termination. But mechanisms of protein synthesis in yeast and high eukaryotes could be different. We cloned human homolog of Dbp5 (Ddx19) and produced recombinant protein in E. coli. Helicase activity of recombinant human Dbp5 was demonstrated using luciferase based assay. By gel filtration we have shown an ability of human Dbp5 to bind eRF1 in the presence and absence of ATP in vitro, but only 20% of both proteins assembled into complex. We suppose that for effective Dbp5-eRF1 complex formation additional factors are required. Activity of human Dbp5 in translation termination was tested in reconstituted in vitro eukaryotic translation system. We have shown appearance of the additional toe-print peak during termination. Probably binding of eRF1 with Dbp5 during translation changes conformation of ribosomal complex and regulates peptide release.
One remaining question in the early life history is to understand how evolution could have passed from complex prebiotic chemistry to simple biology. Current cellular facts allow us to follow the link between chemical then biochemical metabolites from ancient to current world. Accordingly, water, metals as well as simple organics provide a window into the union of the inorganic/organic and biological worlds.

In this context ‘RNA world’ hypothesis proposes that early in the evolution of life, RNA was responsible both for the storage and transfer of genetic information and for the catalysis of biochemical reactions. Metals as well as water dependence activity of various natural hammerhead and hairpin ribozymes are explored showing how the catalysis depends on the surrounding biochemical conditions. For instance, performing the reaction with Ca²⁺ instead of Mg²⁺ reduces the proportion of active molecules. In fact, the dynamic nature of the hammerhead ribozyme where the presence of the loop-loop tertiary interaction and/or high Mg²⁺ concentrations, favors its conformational flexibility instead of locking the ribozyme. On the other hand, the existence of contemporary life in extreme conditions such as diversified abyssal life, conditions which probably occurred during the evolution of life, encourage us to focus on the activity, persistence and dynamics of RNA at extreme temperatures, extreme pH and/or high pressure. Last, studying viroids as plausible remains of ancient RNA, we recently shown the viroid replication in an aspecific host, emphasizing their adaptability to different environments which enhances the survival probability in a primeval auto-replicative system.

References
post-transcriptional modifications of rRNAs in the nucleolus. Box C/D snoRNAs are assembled with a set of four core proteins. Their assembly occurs in the nucleoplasm but the mechanisms involved are not yet clearly defined although we know several assembly factors such as Nufip and the HSP90/R2TP system, which contain the key AAA+ ATPases Rvb1 and Rvb2. Here, we described a new snoRNP assembly factor named Trip3. We showed that Trip3 dimerizes with Nufip and is important for first steps of snoRNP assembly. By quantitative SILAC proteomics, we found that Nufip/Trip3 associates with part of the R2TP complex and some snoRNP core proteins to form a complex devoid of RNA. This complex is subsequently recruited on nascent box C/D snoRNA, and this occurs concomitantly with removal of Trip3 while Nufip is removed at a later stage. By performing detailed mutagenesis coupled to NMR studies and structural modeling, we obtained a model of the interaction of a fragment of Nufip with pre-snoRNP. This model shows that Nufip keeps pre-snoRNPs in an inactive state, and that Nufip must be removed to allow formation of the active snoRNP structure. We propose that this is catalyzed by the AAA+ ATPases Rvb1/2.

**SW01.S2–35**

**Characterization of the pre-60S ribosomal particles able to translate in *Saccharomyces cerevisiae***

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Ribosomes are large ribonucleoprotein particles that are composed of two subunits (40S and 60S in eukaryotes). Ribosomes assemble in a highly dynamic process that involves 79 ribosomal proteins, four rRNAs, more than 200 protein factors and about 100 snoRNAs. All these factors, whose precise functions are still largely unknown, likely allow the ribosome maturation process to proceed with the required speed, accuracy and directional-ity. Ribosome biogenesis starts in the nucleolus, continues in the nucleoplasm and is completed after nucleo-cytoplasmic export of the subunits in the cytoplasm.

In wild type conditions, pre-rRNA processing is coordinated with the nucleo-cytoplasmic export of pre-ribosomal particles. During 60S maturation, 7S pre-rRNAs are 3′ processed to 6S by the nuclear exosome; 6S pre-rRNAs are processed to mature 5.8S rRNAs in the cytoplasm. Only those pre-60S particles containing 6S pre-rRNAs are actively exported to cytoplasm. In any case, immature pre-60S particles are translating inactive.

Our group is interested in understanding how yeast 60S ribosomal subunits achieve translation activity. We have observed that in some mutant backgrounds, the pre-60S particles contain- ing 5.8S + 30 pre-rRNAs and 7S pre-rRNAs are actively exported to the cytoplasm. More interesting, these immature pre-60S particles fractionate in the polysome region of sucrose gradients, hence, strongly suggesting that they are able to translate. Experiments are in progress to define the features that allow these pre-60S particles to escape the nuclear retention mechanism and engage into translation.

**SW01.S2–36**

**EBV encoded miR-BART15-3p promotes cell apoptosis by targeting BRUCE***

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Epstein–Barr Virus (EBV) generates a variety of viral miRNAs by processing the BHRF1 and BART transcripts. BART miRNAs are expressed in all EBV latent infected cells, but the functions of the majority of BART miRNAs are still unknown. We found that miR-BART15-3p promoted apoptosis by itself and potentiated the apoptotic effect of 5-FU by FACS analysis following Annexin V or Propidium iodide staining. Furthermore, the inhibitor for miR-BART15-3p reduced apoptosis of EBV infected cells. Using bioinformatics analysis, we predicted that miR-BART15-3p may target anti-apoptotic BCL2, BCL2L2, DDX42 and BRUCE mRNAs. Luciferase reporter assay showed that only the 3′-UTR of BRUCE was affected by miR-BART15-3p, while the others were not affected. There are two putative seed matched sites for miR-BART15-3p on BRUCE 3′-UTR. Mutation study supported that miR-BART15-3p hybridized only with the first seed matched site on the BRUCE 3′-UTR, miR-BART15-3p down-regulated BRUCE protein, without affecting the BRUCE mRNA level. Our data suggest that miR-BART15-3p induces apoptosis by inhibiting translation of an inhibitor of apoptosis, BRUCE. Further study is warranted to understand the role of miR-BART15-3p in the EBV life cycle.

**SW01.S2–37**

**Guard and protect versus seek and destroy the antisense: contrasting roles of Hfq and PNPase in the regulation of non-coding RNAs***

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Bacterial small RNAs (sRNAs) are extremely labile when are not associated with the RNA chaperone Hfq. This protein is homolo-gous to Sm and Sm-like proteins involved in RNA processing in eukaryotes and it facilitates the sRNA-mRNA annealing. Although critical for sRNA stabilization, the mechanism by which Hfq stabilises sRNAs has been elusive. Through our mutagenic studies we have defined distinct modules in the regulatory MicA RNA [1]. Particularly, two different Hfq-binding regions were identified: an internal A/U-rich sequence and the 3′ end poly(U) tail. In the absence of Hfq, all sRNAs tested were slightly shorter than their full-length species as result of 3′-end trimming. Here we show that the Hfq-mediated protection of the 3′ end is critical for the sRNA stability.

The 3′–5′ exonuclease polynucleotide phosphorylase (PNPase) is the major nuclease involved in the rapid degradation of small RNAs, especially those that are free of binding to Hfq [2]. The levels of several non-coding RNAs are drastically increased upon PNPase inactivation. The turnover of Hfq-free small RNAs is growth phase regulated and PNPase degradative activity is particularly important in stationary-phase. Indeed, the exonuclease PNPase makes a greater contribution than the endonuclease RNase E, commonly believed to be the main enzyme in the decay of sRNAs. Our data also suggests that when not associated with Hfq, the sRNA-degradation occurs mainly in a target-independent pathway in which RNase III has a reduced impact.

The small RNA degradation by PNPase and the counter protection offered by Hfq seems far more common in the living world than was previously envisaged. Our data challenges the model of sRNA degradation and contributes to the development...
of new strategies to program gene expression networks through sRNA biology.

References

SW01.S2–38
Phylogenetic analysis of bacterial group II intron-encoded ORFs
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Group II introns are self-splicing RNAs that act as mobile retroelements in the organelles of plants, fungi and protists. They are also widely distributed in bacteria, and are generally assumed to be the ancestors of nuclear spliceosomal introns. Most bacterial group II introns have a multifunctional intron-encoded protein (IEP) ORF within the ribozyme domain IV (DIV). This ORF encodes an N-terminal reverse transcriptase (RT) domain, followed by a putative RNA-binding domain with RNA splicing or maturation activity and, in some cases, a C-terminal DNA-binding (D) region followed by a DNA endonuclease (En) domain. In this study, we focused on bacterial group II intron ORF phylogenetic classes containing only reverse transcriptase/maturase open reading frames, with no recognizable D/En region (classes A, C, D, E, F and unclassified introns). On the basis of phylogenetic analyses of the maturase domain and its C-terminal extension, which appears to be a signature characteristic of ORF phylogenetic class, with support from the phylogeny inferred from the RT domain, we have revised the proposed new class F, defining new intron ORF varieties. Our results increase knowledge of the lineage of group II introns encoding proteins lacking the En-domain.

Reference

SW01.S2–39
Long terminal stem increases the lifetime of small non-coding RNAs in mammalian cells
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Two small (about 100 nt) non-coding nuclear RNAs (4.5SH and 4.5SI) with unknown functions are synthesized by RNA polymerase III, present in various tissues, and have relatively narrow taxonomic distribution (four and three rodent families, respectively). We found that 4.5SH RNA is short-lived (t1/2 approximately 18 min) whereas 4.5SI RNA is long-lived (t1/2 approximately 22 h). Then we searched for RNA structural features that determine the short lifetime of 4.5SH in comparison with the long lifetime of 4.5SI RNA. Chimeric 4.5SH/4.5SI RNA genes were obtained and human cells (HeLa) were transfected with these constructs. The decay rate of the original and altered RNAs was measured. 4.5SH RNA lifetime increased only when its 5’ and 3’ ends were replaced with terminal sequences of 4.5SI RNA. Secondary structure analyses revealed that both the chimeric RNA and natural 4.5SI RNA had a long 16-nt terminal stem. The replacement of the 3’ end region of 4.5SH RNA with the sequence complementary to its 5’ end region also increased the RNA lifetime. On the other hand, the disruption of the terminal stem in 4.5SI RNA dramatically decreased its lifetime. Thus, the complementarity of end regions of 4.5SI RNA contributes to its stability in cells, whereas the lack of such complementarity in 4.5SH RNA causes its rapid decay.

SW01.S2–40
Alternative splicing of the X-linked NDUFB11 gene and its implication in mitochondrial complex I function and apoptotic process
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Alternative splicing is a critical process in the complexity and function of the eukaryotic genome. Through this mechanism a precursor mRNA, transcribed from a single gene, can be processed to generate alternative splicing transcript, some of which encoding for different proteins. More than one alternative splice isoform can be maintained concurrently in the steady-state mRNA pool of a single tissue or cell type and changes in the isoform ratios have been associated with physiological variation and susceptibility to disease. In this paper we have studied the role of alternative splicing transcripts of Ndufb11 gene in the regulation of mitochondrial complex I function and in the apoptotic process. In physiological conditions the human X-linked Ndufb11 gene produces at high level a short transcript isoform encoding for the 153 aa subunit of the mitochondrial complex I, with respect to a long transcript isoform encoding for the 163 aa protein of unknown function. Treatment of neuronal cells with rotene none affects the ratio of the Ndufb11 isoforms, increasing the relative amounts of the long versus the short transcript. Evidence is presented here showing that over-expression of the 163 aa subunit induced a down-regulation of the complex I enzymatic activity and an overproduction of ROS. Indeed, we have found that in plasmid transfection higher levels of the 163 aa expression compared to the 153 aa protein, lower cell viability. However, the 163 aa subunit transfecants maintained viability and did not undergo apoptosis. On the other hand 163 aa over-expression sensitized the neuroblastoma cells to apoptosis induced by rotene none.
transfection (MATRA) is easy, fast and very effective technology for transfecting cells in culture.

We aimed to determine the expression profiles of 48 genes that were important for leukemia progression and effect on apoptosis in K-562 and NCI-BL 2347 cell lines which transfected with down regulated miR-150 by MATRA mediated non-viral method. For this purpose, chronic myeloid leukemia model cell line; K-562 and non-leukemia cell line NCI-BL2347 were used.

Apoptosis/necrosis detection was analyzed by Annexin V-EGFP and DeadEnd Fluorometric TUNEL methods in the course of 72 h. As a control group, HEPES (10 mM) was used for each cell line. Total RNA was isolated and cDNA synthesis was performed from the cells after 48 h from the transfection, and changes in gene expressions were examined by real time online RT-PCR. GAPDH, ACTB and RN18S1 were used as housekeeping genes.

Apoptosis was induced 17.86 fold (72nd hour) and 1.68 fold (48th hour) in NCI-BL2347 and K562 cells, respectively compared to control cells.

As a result, expressions of TNFαR59, TNF and DAPK1 genes were decreased twofold when compared to the control cells.

In conclusion according to gene expression and apoptosis results, nanoparticle (MATRA)-mediated transfection of miR-150 into leukemia cells could be effective in the treatment of leukemia in vitro.

**SW01.S2–42**

**Novel, 3′-terminal phosphate modification activities of 5′-pRNA ligase from Methanobacterium thermoautotrophicum**

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The RNA ligase from the archaea *Methanobacterium thermoautotrophicum* (MthRnl) is a heat-stable ATP dependent enzyme that can ligate 3′-OH of RNA or ssDNA to a 5′-phosphorylated single-stranded nucleic acid through the mechanism of 5′-phosphate adenylation. We discovered that MthRnl is also able to adenylate synthetic oligonucleotides with terminal 3′-phosphate and either a 2′-deoxy- or 2′-O-Me-modifications, producing ssDNAappA and RNA (2OMe)pA respectively. In addition, MthRnl synthesizes RNA 2′, 3′-cyclic phosphate from 3′-terminal phosphorylated RNA. This activity has the same mechanism and efficiency of cyclization as the ATP-dependent terminal RNA phosphate cyclase (RtcA) from *E. coli* and a similar mechanism of 3′-phosphate activation and modification as the GTP dependent RtcB ligase which can ligate RNA 3′ p to 5′ OH RNA. Comparisons to other RNA ligases revealed that the T4 RNA ligases 1 and 2 do not have 3′-adenylation/2′-cyclization activities. MthRnl and the T4 RNA ligases, cannot ligate 3′-phosphorylated or 2′, 3′-cyclic phosphorylated substrates to the 5′-OH of RNA or ssDNA. While assaying these enzymes we found that 2′, 3′-phosphate cyclization is not strictly required for RtcB ligation activity, but terminal 2′-oxygen is important for ligation. RtcB, in addition to RNA, can act on ssDNA 5′OH substrates as long as the 3′-phosphorylated substrate is RNA. In the absence of 5′OH acceptor the final product of terminal 3′-phosphate modification by RtcB is 2′, 3′-cyclic phosphate. These combined modifications of 3′- and 5′-phosphorylated substrates expand the potential biological roles of RNA ligases and their applications for RNA analysis.

**SW01.S2–43**

**rRNA methyltransferase RsmD Escherichia coli**

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Ribosomal RNA is modified in all living organisms. Modified nucleotides are clustered in the most functionally important centers of the ribosome: ligand-connecting areas, peptide-transferase centre, places of contact of two subunits. The majority of modified nucleotides in *Escherichia coli* rRNA are various types of base and ribose methylated residues. Ribosomal RNA modification is accomplished by a variety of enzymes acting on all stages of ribosome assembly. Some of such enzymes could act on completely deproteinized *rRNA in vitro*, while others could modify assembled subunits [3], some have preferences for assembly and, among them, one protein that prefers associated 70S ribosomes [1]. Our study is devoted to a very unusual enzyme—rRNA methyltransferase RsmD. The target of this enzyme, nucleotide G966 of the 16S rRNA, is located in a deep cleft of the 30S subunit, which is occupied by a P-site bound rRNA anticodon [2]. RsmD acts late in the assembly process and is able to modify a completely assembled 30S subunit. It possesses superior binding properties toward the unmodified 30S subunit but is unable to bind a 30S subunit modified at G966. Dissociation constants for RsmD complexes with unmethylated 30S subunits were found to be in the 30–40 nM range. The dissociation constant for SAM binding to RsmD, 0.58 mM, is comparable to or slightly better than that of other rRNA methyltransferases. RsmD is unusual in its ability to withstand multiple amino acid substitutions of the active site. Such efficiency of RsmD may be useful to complete the modification of a 30S subunit ahead of the 30S subunit’s involvement in translation [3].

**References**


**SW01.S2–44**

**3′UTR-mediated rhythmic translation of Cry1**

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We investigated the 3′ untranslated region (UTR) of the mouse core clock gene *cryptochrome 1 (Cry1)* at the translational level. Interestingly, the 3′UTR of Cry1 decreased its mRNA levels but increased protein amounts. The 3′UTR is widely known to function as a cis-acting element of mRNA degradation, and 3′UTR mainly suppresses translation of target mRNAs. We found that heterogeneous nuclear ribonucleoprotein D (HNRNP D) rhythmically binds to the Cry1 3′UTR and regulates translation of Cry1 mRNA in time-dependent manner. HNRNP D interacted with eukaryotic translation initiation factor 3 subunit B (EIF3B) and also directly associated with ribosomal protein S3 (RPS3) or RPS14, resulting in translation of Cry1 in a 3′UTR-dependent manner. Our results suggest that the 3′UTR is important for
rhythmic translation, and HNRNPD bound to the 3′ UTR facilitates interaction with the 5′ end of mRNA by interacting with translation initiation factors and recruiting the 40S ribosomal subunit to initiate translation.

**SW01.S2-45**

Comprehensive analysis of artificial box C/D RNAs action on human cells

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Box C/D small nuclear RNAs (snoRNA) are commonly known to guide the site-specific 2′-O-ribose methylation of nucleotides in eukaryotic RNA, such as rRNA and snRNA. Recently snoRNAs are predicted to regulate post-transcriptional modifications of pre-mRNA. The object of our study was to expand the understanding of the role of small nuclear box C/D RNAs and snoRNA-like transcripts in regulation of gene expression in human cells.

Artificial U24 box C/D snoRNA analogues directed to nucleotides of pre-mRNA, and mature mRNA HSPA8 (encoding human heat shock cognate protein 70, hsc70) are designed and synthesized. Artificial RNAs contained target recognition regions directed to following pre-mRNA nucleotides: the branch point adenosine; the first and the last nucleotides of the second intron; the donor and the acceptor nucleotides of the spliced exons. Level of mRNA HSPA8 was determined with real time RT-PCR method. Furthermore we analyzed transcriptome of MCF-7 cells transfected by artificial snoRNAs with Illumina HT microarrays.

We found that transfection of the artificial analogues into MCF-7 human cells suppressed the HSPA8 mRNA level. The decreased yield of the major product of HSPA8-specific RT-PCR was accompanied by increased yield of minor products corresponding to aberrant (shorter) products of HSPA8 pre-mRNA splicing, suggesting that box C/D RNA analogues impaired the splicing of target pre-mRNA. Moreover the transfection with artificial box C/D RNAs was found to decrease human adenocarcinoma (MCF-7 and MDA-MB-231) cell viability. The microarray analysis followed by RT-PCR analysis showed reliable upregulation of interferon-induced genes indicating that artificial box C/D RNAs caused the innate immune response of human cells, with STAT1-dependent gene level increasing.

The study provides a novel tool to offer insights into the question of whether snoRNAs that are complementary to pre-mRNAs could affect the processes of target pre-mRNA maturation. Our results showed that transfection of human cells with artificial snoRNAs containing guide sequences directed to pre-mRNA could impair the splicing and affect overall mRNA isoform levels. Transfection with artificial snoRNAs decreased human cell viability, suggesting that snoRNA analogues are able to activate regulatory processes affecting vital cellular functions.

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**SW01.S2-46**

Interaction of *Bacillus subtilis* 6S-1 and 6S-2 RNAs with RNA polymerase – comparative functional analyses and proteomics of 6S-1/2 knockout strains

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6S RNAs are a new type of regulator – small non-coding RNAs that are able to inhibit transcription by direct binding to the bacterial RNA polymerase holoenzyme (RNAP), thereby blocking interaction with DNA promoters [1]. Under certain conditions 6S RNA can serve as a template for the transcription of short abortive ‘product RNAs’ (pRNAs, 14–24 nt), which stay bound to 6S RNA and lead to dissociation of 6S RNA:RNAP complexes and possibly 6S RNA degradation [2]. In contrast to *E. coli* and many other bacteria encoding a single 6S RNA, the *B. subtilis* and related species encode two 6S RNA homologs, termed 6S-1 (bsrA) and 6S-2 (bsrB) [3], which are most abundant in late exponential/stationary phase or exponential phase, respectively. Recent investigations provided evidence that 6S-1 RNA displays all hallmarks of a canonical 6S RNA [3], while the function of the 6S-2 RNA in *B. subtilis* has remained as yet elusive.

We investigated the functional properties of *B. subtilis* 6S-2 RNA in direct comparison with those of 6S-1 RNA. *In vitro* transcription assays showed that both 6S RNAs specifically inhibit transcription at model DNA promoters with comparable efficiency. This result is in line with very similar affinities of both 6S RNAs to the housekeeping σ^A^-RNAP. Furthermore, 6S-2 RNA can act as an efficient template for pRNA synthesis in *vitro*. Such short transcription products (approximately 13–16-mers) form 6S-2:pRNA hybrids, which dissociate much more rapidly than the major long 6S-1 pRNA variant (14-mer) from their respective 6S RNA template. However, longer variants of pRNA_{6S-2} (20-mer) remain stably bound to 6S RNA and prevent its binding to RNAP. To elucidate the physiological role of 6S-2 RNA we performed proteomic analyses of bsrA and bsrB knockout strains in comparison with the wild-type. Substantial changes in the protein pattern were detected for both 6S RNA knockout strains. Several proteins, whose expression was demonstrated to be affected by the absence of 6S-1/2 RNA, were identified by MALDI mass spectrometry.

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**References**


Tandem repeats (TRs) constituted by continuous repeating sequences are an important source of genetic variation. Previous studies have linked variation in repeat length with phenotypes such as human diseases and canine skull morphology [1–3]. However, an integrated understanding of how TRs influence the flow of genetic information determining the abundance and function of mRNA and the encoded proteins, to affect phenotypes, under normal physiological conditions, has remained elusive. Through the investigation of multiple genome-wide datasets we provide insights into how TRs in the coding regions of mRNAs and in proteins affect different regulatory steps involved in mRNA and protein-expression homeostasis in Saccharomyces cerevisiae. Cells achieve the regulation of availability of molecules by a fine balance between synthesis and degradation. At the mRNA level, we show that genes with multiple TRs in the coding regions have higher transcript abundance, owing to higher half-life. The increased tendency of these transcripts to form secondary structures leads to slower degradation. In contrast, proteins with aminoacid TRs are less abundant owing to lower translational rates, and shorter half-life compared to those without TRs. This tight regulation of TR proteins is more pronounced for proteins with polar uncharged aminoacid TRs such as polyGln. Thus the presence of TRs confers stability to transcripts, creating a reservoir at the mRNA level while a coordinated, tight regulation of TR proteins at several stages during translation ensures that they are present for short amounts of time and in low quantities. This strategy minimizes the harmful effects of TR proteins, which are important regulators of gene expression, simultaneously permitting their vital contribution to the functioning of a cell. Deriving such general principles aids in determining the effect of repeat length variation on diverse molecular processes influencing phenotypes, especially human diseases.

References

Identification of molecular targets of RNase A in antitumor therapy
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In recent years, ribonucleases are regarded as perspective anticancer drugs. Novel data, suggesting an important role of miRNAs in mediating tumor growth and invasion, have provided researchers with a new area to search for possible molecular targets of cytotoxic ribonucleases. Previously, antitumor and antimetastatic properties of pancreatic ribonuclease RNase A have been verified (Patutina O.A. et al., Biochimie 2011). It was found that observed tumorcidal activity of RNase A was accompanied by a reduction in pathologically elevated levels of extracellular RNAs and an increase in ribonuclease activity of blood plasma of tumor-bearing animals. In the present study, by high-throughput SOLiD sequencing technology we performed an analysis of genome-wide profiles of miRNAs in tumor and serum of mice after treatment with RNase A. Sequencing data revealed that RNase A therapy resulted in an apparent alteration in the levels of 215 serum and tumor-derived miRNAs. Analysis of miRNA expression profile showed that the pool of miRNA with most significantly changed expression level contained a considerable number of ascertained tumor-associated miRNAs, such as miRNAs from let-7 family, mir-21, mir-10b, mir-145, mir-451a, mir-29b1, mir-17 and others. RNase-mediated alteration of mir-10b, mir-145a, let-7g and mir-451a, miR-31, miR-21 was validated by qRT-PCR. Changes in miRNA expression were attended by an upregulation of tumor and metastasis suppressor genes Pten, Timp3, CD82 and Brms1. The obtained data give the evidence that antitumor and antimetastatic effects of RNase A is associated with alteration in miRNA profiles in tumor tissue and blood serum of animals promoting the strengthening of adhesive properties of tumor cells and impeding an ability to metastasize.

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Differential expression of microRNAs in plasma of patients with laryngeal squamous cell carcinoma: potential early detection markers for laryngeal squamous cell carcinoma
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The aim of the study is to investigate the alterations of plasma microRNAs (miRNA) miRNAs in laryngeal squamous cell carcinoma (LSCC). Altered miRNA expression has been found in many cancers, including lung, breast, prostate, bladder and colorectal cancer. Many recent studies have demonstrated that aberrant plasma miRNAs were also found in various types of cancers. However, the alteration of plasma miRNA expressions in LSCC remains unclear. In this present study, the expression profiles of 738 miRNAs in plasma from 20 patients and 44 healthy subjects were evaluated using high-throughput real-time quantitative polymerase chain reaction (qRT-PCR). Our results demonstrated that expression levels of 17 miRNAs were significantly up-regulated in LSCC patients when compared to control group (p < 0.05). Expression levels of nine miRNAs were found significantly down-regulated in LSCC patients (p < 0.05). In addition, 17 miRNAs were expressed only in LSCC group and five of these miRNAs (miR-331-3p, 603, 1303, 660-5p and 212-3p) are LSCC specific and never seen before in plasma of any human subject.

In conclusion, our study suggests that detecting these LSCC specific miRNAs in plasma might serve as novel non-invasive biomarkers for LSCC.
Circulating non-coding RNAs as biomarkers of head and neck cancers

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Abstract: Head and Neck Squamous Cell Carcinoma (HNSCC) is the 6th common malignancy in men. We currently have little understanding of the molecular events of this disease and no biomarkers currently exist for early detection. Recently, small non-coding RNAs such as microRNAs (miRNAs) were shown to be highly stable and could be found in body fluids such as serum. Given this, circulating miRNAs found in the blood of HNSCC patients could act as potential clinical biomarkers for early detection.

Methods: Using Agilent miRNA arrays we screened for the expression of circulating miRNAs in patient sera (n = 52) showing the four representative subtypes of HNSCC and in sera isolated from normal individuals (n = 11). A number of candidate miRNAs biomarkers were identified and validated using TaqMan qPCR. These biomarkers were then assessed for clinical relevance.

Results: Ninety-three dysregulated serum miRNAs were identified across all tumours in comparison to healthy sera. Specifically, levels of serum miRNAs can distinguish four different subtypes of HNSCC and in sera isolated from normal individuals (n = 11). A number of candidate miRNAs biomarkers were identified and validated using TaqMan qPCR. These biomarkers were then assessed for clinical relevance.

Conclusions: Our study is the first to show that the expression levels of serum miRNAs can distinguish four different subtypes of HNSCC. QPCR analysis supported these findings with further studies now being validated in a larger cohort of clinical samples. Our findings provide a promising foundation for the application of small RNAs as biomarkers for the early detection of HNSCC.

Small non-coding RNAs of human blood plasma of healthy donors and patients with non-small cell lung cancer

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Abstract: Circulating nucleic acids are the subject of numerous current investigations aimed to establish novel mechanism of distant regulation of physiological processes, and to utilize extracellular nucleic acids as diagnostic and prognostic markers of pathological processes in the organism.

The purpose of this study is a detailed description of structure and forms of short extracellular RNAs of human plasma, aimed at identifying new forms of regulatory RNA and the development of unique and complex diagnostic markers of human diseases.

In this study we analyze the diversity of short non-coding blood plasma RNA of eight healthy volunteers and eight patients with non-small cell lung cancer. In order to obtain cDNA libraries that encode the most full-scale set of circulating RNAs, short (n > 19) plasma RNAs were exposed to dephosphorylation followed by 5'-phosphorylation, ligation with adapters, reverse transcription and amplification. Individual cDNA libraries were sequenced on SOLiD platform (V.3). The resulting data sets were analyzed using the Bowtie/Cufflinks software.

Background: Non-small cell lung cancer (NSCLC) is the 6th common malignancy in men. We currently have little understanding of the molecular events of this disease and no biomarkers currently exist for early detection. Recently, small non-coding RNAs such as microRNAs (miRNAs) were shown to be highly stable and could be found in body fluids such as serum. Given this, circulating miRNAs found in the blood of NSCLC patients could act as potential clinical biomarkers for early detection.

Methods: Using Agilent miRNA arrays we screened for the expression of circulating miRNAs in patient sera (n = 52) showing the four representative subtypes of NSCLC and in sera isolated from normal individuals (n = 11). A number of candidate miRNAs biomarkers were identified and validated using TaqMan qPCR. These biomarkers were then assessed for clinical relevance.

Results: Ninety-three dysregulated serum miRNAs were identified across all tumours in comparison to healthy sera. Specifically, levels of serum miRNAs can distinguish four different subtypes of NSCLC and in sera isolated from normal individuals (n = 11). A number of candidate miRNAs biomarkers were identified and validated using TaqMan qPCR. These biomarkers were then assessed for clinical relevance.

Conclusions: Our study is the first to show that the expression levels of serum miRNAs can distinguish four different subtypes of NSCLC. QPCR analysis supported these findings with further studies now being validated in a larger cohort of clinical samples. Our findings provide a promising foundation for the application of small RNAs as biomarkers for the early detection of NSCLC.

Sequence-specific endonucleonuclease from PemikSa toxin-antitoxin system modulates gene expression in Staphylococcus aureus

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Abstract: Numerous regulatory mechanisms in bacteria, which control the response to environmental conditions, were discovered. Recently, the idea of toxin-antitoxin (TA) systems as potential gene expres-
sion regulators has emerged. This study is focused on the pemIKSa TA system of Staphylococcus aureus strain CH91. The toxin PemKSa is sequence-specific endoribonuclease toward tetrad sequence UAUU as determined in vitro using the primer extension method. A set of expression experiments in Escherichia coli, based on recombinant plasmids, confirmed the sequence-specific degradation of transcripts and following suppression of the production of corresponding proteins. Moreover, computational studies revealed that the UAUU sequence in underrepresented in a substantial number of transcripts encoding proteins implicated in staphylococcal virulence whereas the target sequence is overrepresented in gene transcripts coding for transmembrane transporters. The nonrandom distribution of the PemKSa target sequence in the staphylococcal transcriptome, and significant functional consistency within both groups of above transcripts, argue in favor of the hypothesis of a regulatory role for PemKSa. To this end, the investigation of the pemIKSa TA system properties in the native host, the S. aureus has been performed. Introduction of a recombinant plasmid carrying pemKSa gene under the control of an inducible promoter showed strong bacteriostatic properties of the PemKSa toxin. Analysis of the mRNA pool showed the reduction in the amount of transcripts of genes encoding transcription factors, quorum sensing system components and transmembrane transporters whereas some transcripts of house-keeping genes and those coding for factors implicated in staphylococcal virulence remain unaffected. The obtained results strongly suggest that the pemIKSa system possesses properties of a gene expression regulator. Moreover, the ability to induce bacteriostatic effect implies its possible role in a response to adverse environmental conditions.

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SW01.S2-54
A strategy of isolation of telomerase from yeast Hansenula polymorpha
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Telomerase sustains telomere homeostasis and provides unlimited proliferative potential for unicellular eukaryotes, as well as stem, germ and cancer cells. The two core components of telomerase complex are telomerase RNA (TER) and telomerase reverse transcriptase (TERT). Functional and structural characterization of the telomerase is compromised by difficulties in obtaining sufficient amount of pure complex.

Our group is developing a strategy of isolation of telomerase from thermotolerant yeast Hansenula polymorpha. Here we describe telomerase RNA of H. polymorpha (HpTER) tagged with an RNA hairpin, which is a binding target of the coat protein of PP7 phage. Different sites of PP7 hairpin insertion were tested on the functionality of tagged HpTER in vivo and the ability to recover HpTER from yeast extracts during affinity purification procedure.

SW01.S2–55
Highly specific transcription templates and sensors for RNA polymerase activity based on single-stranded DNA aptamers
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Bacterial RNA polymerase (RNAP) is known to recognize single-stranded nucleic acid templates of various nature, resulting in synthesis of either abortive or productive RNA transcripts. To reveal the general recognition principles and the mechanisms of transcription initiation on single-stranded templates we obtained highly-specific ssDNA aptamers to RNAP holoenzyme. The aptamers have a hairpin structure that partially mimics the structure of the open promoter complex with properly placed –10 and TG promoter elements. Subtle modification of the aptamer structure converted them into efficient transcription templates, specifically recognized by RNAP holoenzyme. Depending on the downstream template structure and the presence of consensus promoter elements, we obtained template variants capable of synthesis of RNA transcripts of various lengths and types. Thus, the aptamer part can be used as a recognition module for specific initiation of RNA synthesis on ssDNA templates of various structures and origins. Modification of the aptamer templates with specifically positioned fluorescent and quenching groups allowed to obtain sensors for fluorescence-based measurements of the RNA polymerase activity. The aptamer-based templates provide a novel tool for structure-function analysis of transcription initiation and for development of efficient single-stranded templates and transcription sensors.

SW01.S2–56
Effects of RapA, the bacterial SWI2/SNF2 family factor, on transcription in vivo
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The SWI2/SNF2 family consists of a large number of proteins present in all kingdoms of life. The most studied eukaryotic SWI2/SNF2 members are involved in ATP-dependent remodeling of DNA and RNA-protein complexes and play roles in the regulation of gene expression. Escherichia coli SWI2/SNF2-like protein RapA is an RNA polymerase (RNAP) associated factor. RapA is as abundant in the cell as RNAP subunit sigma-70 which competes with RapA for the binding to the core of RNAP. Previous studies of RapA were restricted by in vitro works and it was demonstrated that RapA stimulates transcription by facilitating RNAP recycling. However, the actual mechanism of RapA operation in vivo and its physiological functions are poorly understood.

To test the effect of RapA on transcription in living cells, we used an in vivo assay to monitor the behavior of transcribing RNAP in two isogenic E. coli strains, wild type and rapA(-) mutant. We constructed a plasmid in which RNAP initiates transcription from a constitutive promoter hisR and terminates at trp intrinsic terminator. The elongation properties of RNAP were investigated by in situ footprinting of the transcription bubble with the single-strand specific probes, potassium permanganate or chloroacetalddehyde. Analysis of the probes modifications on the non-template strand and their changes after addition of rifampicin, which blocks new rounds of transcription, revealed a significant difference in the reactivity of DNA region in the vicinity of the thymidine (T) stretch of the terminator in each strain. Clearly, RNAP remains at the T stretch in rapA(-) strain for a longer time than RNAP in wild type strain. To study pausing at the intrinsic terminator, the template was modified so that multiple substitutions in the upstream half of the termination hairpin prevented hairpin formation. We found even more reactivity at the T stretch in rapA(-) strain than in wild type that obviously reflects the influence of RapA on non-productive RNAP at this site. Our results demonstrate that RapA promotes efficient in vivo transcription by remodeling trapped/paused elongation complexes at the intrinsic terminator.

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Identification and differential expression analysis of conserved and novel microRNA in flax genotrophs

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Flax (Linum usitatissimum L.), is important agricultural crop. Flax stem fiber and seed oil have multiple industrial applications. Flax undergoes heritable changes in phenotype and genotype in response to the growth environment [1]. Flax lines in which stable inherited changes were observed were named genotrophs. Very little is known about mechanisms of genetic changes during flax development under defined environment conditions. Small non-coding RNAs are a distinct class of regulatory RNAs in plants that control a variety of biological processes. Small RNAs regulate growth and development, maintenance of genome integrity, and are also important components in plant stress responses.

The comparison of miRNA expression profiles was performed for flax cultivar Stormont Cirrus grown under normal (1), inadequate (2) and excessive (3) nutrition using the miRNA database (Suppl. 1) (2013) 3–617.

A total of 7.2 M (1), 11.6 M (2), and 7.6 M (3) raw reads were obtained from deep sequencing of flax sRNA. The highest read abundance was found for 24-nt (over 25%) and 21-nt (23%) sRNAs. We identified 76 conserved miRNA homologues which belong to 20 distinct miRNA families. The highest read abundance was detected for miR166 family. In total, we predicted 50 potential novel miRNAs for (1), (2) and (3) libraries respectively. Some of the conserved and novel miRNAs showed differential expression among (1), (2) and (3) libraries.

Thus, we identified conserved and novel miRNA that were expressed in flax seedlings and perform expression analysis of miRNA in flax genotrophs. It gives new information about the processes occurring while the plants were growing under different conditions and the role of the environment in generating adaptive mutation.

Some of the conserved and novel miRNAs showed differential expression was detected for miR166 family. In total, we predicted 50 potential novel miRNAs for (1), (2) and (3) libraries respectively. Some of the conserved and novel miRNAs showed differential expression among (1), (2) and (3) libraries.

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To investigate the possible role of SncmtRNA and ASncmtRNAs.

Mitochondrial non-coding RNAs (ncRNAs) expression in Human Papilloma Virus-related cervical lesions

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Cervical cancer is the second most common neoplasm in women worldwide and the role of HPV (Human Papilloma Virus) oncogenes in cellular immortalization and transformation has been widely investigated. Recently, studies have shown that normal cells express a sense mitochondrial ncRNA (SncmtRNA) that seems to be required for cell proliferation and two antisense transcripts (ASncmtRNAs).

To investigate the possible role of SncmtRNA and ASncmtRNAs (-1 and -2) in cervix pathology, we analyzed RNA expression in HPV-induced lesions. DNAs were isolated from cervical specimens (using High Pure PCR Template, Roche Diagnostics) from 60 women (29–65 years old) with different cytology and tested using Linear Array HPV Genotyping Test (Roche Diagnostics). RNAs were isolated (Trizol reagent, Invitrogen) and mitochondrial ncRNAs expression was performed using in house designed primers.

In women with normal cytology and ASCUS (Atypical Squamous Cells of Undetermined Significance), hr(high risk)HPV was absent. On the other hand, 72.2% (13/18) LGSIL (Low-Grade Intraepithelial Lesion) and 93.3% (14/15) HGSIL (High-Grade Intraepithelial Lesion) patients presented infection with hrHPV in single or co-infection represented mainly by HPV16, 18, 31 and 33. We detected differential expression of mtcRNAs between HPV-positive and HPV-negative patients regardless of cytological diagnosis. SncmtRNA and ASncmtRNAs were expressed in ASCUS and normal cells (HPV negative). In striking contrast, tumor cells from cancer patients expressed the SncmtRNA whereas ASncmtRNAs were significantly down-regulated (p < 0.05). ASncmtRNAs were also down-regulated in 76.9% of LGSIL HPV positive patients and in 92.8% HGSIL HPV positive patients.

Down regulation of the ASncmtRNAs seems to be an important step on neoplastic transformation and cancer progression. MncRNAs may represent cofactors in HPV-induced cervical lesions and cancer but their role in the mechanism of carcinogenesis remains to be solved.
Degradome sequencing reveals an endogenous microRNA target in *C. elegans*

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*C. elegans* microRNAs (miRNAs) bind to partially complementary sequences in the 3′ untranslated region of target mRNAs, resulting in translational repression through mRNA destabilization. High-throughput sequencing of RNA cleavage fragments was performed to directly detect miRNA-directed cleavage targets in adult stage *C. elegans*. From this analysis, we found that miR-249 directed the cleavage of the ZK637.6 transcript with extensive and evolutionarily conserved complementarity in nematode. In addition, expression of the ZK637.6 transcript was strongly dependent on the expression of miR-249. These findings may lead to a better understanding of miRNA-mediated gene regulation in nematodes.

An integrated view on genetic and epigenetic mechanisms revealed aberrant DNA methylation as an important source for miRNA deregulation in prostate cancer

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Prostate cancer (PCa) is the most common malignant tumor in males and the third leading cause of cancer-related deaths in Western developed countries. The clinical spectrum of PCa ranges from indolent tumours requiring no therapy to highly aggressive and often metastatic diseases. This clinical heterogeneity has its foundation in the complexity of genetic and epigenetic alterations which are now uncovered in the roam of the International Cancer Genome Consortium (ICGC) projects as well as other cancer genome characterization projects focusing on PCa. It is now well accepted that epigenetic alterations including histone modifications and DNA methylation participate in creating altered gene expression patterns. MicroRNAs (miRNAs) have emerged as important regulators in human tumorigenesis and tumor progression during the last decade. miRNA expression is strongly deregulated in many human cancers including PCa. We investigated the contribution of genetic and epigenetic events to the deregulation of miRNAs in PCa by utilizing the profiling data of the International Cancer Genome Consortium (ICGC) project on Early-Onset Prostate Cancer (http://www.icgc.org). In order to determine to which extent genetic and epigenetic aberrations contribute to miRNA deregulation, an integrative analysis was performed on 19 early-onset PCa comprising genome-wide miRNA expression, DNA structural variation (copy number variation, translocation), single nucleotide variant (SNV) and DNA methylation data within our ICGC project (ICGC EOPCA dataset). We found large-scale epigenetic alterations including both hyper- and hypomethylation of miRNA regulatory regions leading to miRNA silencing or activation, respectively. To confirm this we utilized an independent dataset of PCa with a classical age distribution. Genome-wide miRNA expression data were generated by quantitative PCR screening (qPCR) on 50 prostate cancer and 48 normal prostate tissue samples for which genome-wide methylation obtained by MeDIP has recently been published by Börn et al. Many of the miRNAs, which we prove to be epigenetic regulation by different PCa datasets and validation studies, target genes involved in PCa key pathways mainly PI3K/AKT/PTEN signaling providing further clues on the molecular defects in PCa.

Regulation YB-1 synthesis by mTOR signaling pathway

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The eukaryotic DNA/RNA binding protein YB-1 (Y-box binding protein 1) performs a wide variety of functions at the subcellular level. It participates in pre-mRNA synthesis and splicing, mRNA packaging and regulation of mRNA stability and translation. Also, YB-1 is involved in DNA repair. On the whole, the multifunctional character of YB-1 underlies its important role in cell proliferation, differentiation, stress response, and neoplastic transformation. However, the mechanisms regulating YB-1 synthesis are still known insufficiently.

In this study we have demonstrated that YB-1 synthesis depends on the rate of cell division and is specifically suppressed by inhibition of the mTOR signaling pathway with the inhibitor PP242, but not rapamycin. Experiments on reporter constructs show that dependence of YB-1 mRNA translation on activities of the mTOR signaling pathway is dictated by the 5′ untranslated region (UTR) of this mRNA. Our results suggest that the mechanism of YB-1 synthesis inhibition may involve specific interaction of YB-1 itself with YB-1 mRNA 5′ UTR.

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A novel mechanism of poly A(+) YB-1 mRNA translation regulation

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YB-1 is a DNA- and RNA-binding protein. It is involved in many cellular events. Obviously, its amount in the cell must be closely controlled. As shown recently, translation of polyA(−) YB-1 mRNA is selectively regulated by YB-1 and poly(A) binding protein (PABP) through their specific binding to a regulatory element (RE) within the 3′ untranslated region (UTR) of YB-1 mRNA. YB-1 inhibits its own translation, while PABP stimulates it through displacement of YB-1 from RE.

This study was focused on the effect of these two proteins on translation of poly A(+) YB-1 mRNA. As shown, YB-1 inhibits translation of poly A(+) YB-1 mRNA at the same level as polyA(−), while PABP has no positive effect on translation of this mRNA. This apparently suggests formation of a ‘mini-loop’ (poly(A) tail – proteins – RE) at the 3′-end of the chain. The hypothesis is supported by (i) in vitro formation of a complex between the poly(A) tail and RE in the presence of proteins of rabbit reticulocyte lysate, and (ii) the positive effect of PABP on translation of poly A(+) YB-1 mRNA with a truncated linker between the poly(A) tail and RE. Besides, it has been demonstrated that PABP is necessary but not sufficient for ‘mini-loop’
formation. In other words, more proteins, that are still to be identified, are involved in this process.

Together, these results are evidence for the proposed model of 3'-terminal mini-loop formation and its negative effect on RE-dependent regulation of poly A(-) YB-I mRNA translation by PABP.

SW01.S2–64
Function of Fap7 in the maturation of the ribosome small subunit
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Over 200 pre-ribosomal factors are involved in the maturation of ribosomes. Most of these factors are essential to cell survival, but their precise function remains elusive. One of the last steps of maturation of the ribosome small subunit is the cleavage of 20S pre-rRNA in 18S rRNA in the cytoplasm. This cleavage is carried out by the endonuclease Nob1 and also requires the presence of other factors such as the methyltransferase Dim1, and a plethora of NTPases including the Rio protein kinases, Prp43 and its cofactor Plf1, the Ltv1 GTPase and the Fap7 NTPase.

The function of Fap7 is especially intriguing since the human homologue bears adenylyl cyclase activity, an enzymatic activity not usually found during ribonucleoprotein biogenesis. In addition, the function of Fap7 is intimately linked to its interaction with the Rps14 ribosomal protein. The Rps14 C-terminal domain is essential for D-site cleavage and is located in proximity to the 18S rRNA 3'-extremity in the mature ribosome. The deletion of this protein causes the S4 syndrome that is phenotypically close to Diamond Blackfan anemia. The link between the enzymatic activity of Fap7 and its role in ribosome biogenesis remains enigmatic.

We have conducted functional and structural characterisation of the Fap7 protein alone and in complex with Rps14 and nucleotides. Using a combination of structural studies by X-ray crystallography, small angle X-ray scattering (SAXS) in solution, enzymatic studies on purified proteins, and in vitro D site cleavage reaction assays on purified pre-ribosomes, we were able to uncover the function of Fap7 within pre-40S ribosomes. We show that the Fap7/Rps14 interaction is involved in a major conformational change at the heart of the pre-ribosomes and that this structural rearrangement is necessary to expose the D-site for cleavage by the endonuclease Nob1. The link between the enzymatic activity and the conformational switch both before and after cleavage is currently been investigated.

SW01.S2–65
Molecular mechanisms enhancing the coding potential of RNA genome of influenza A viruses
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Influenza is an acute respiratory illness affecting millions of people worldwide. The epidemiological agents of the disease are Influenza A, B and C viruses of Orthomyxoviridae family. Influenza A virus (IAV) genome consists of eight RNA segments with negative sense polarity. Positive sense mRNAs, from which viral proteins are translated, are synthesized on the basis of these genomic RNAs in the infected cells. Since the small size of the genome (13.5 kb) IAVs have evolved different mechanisms to express multiple viral proteins from a single gene segment. Among them are alternative splicing, alternative open reading frames (ORFs), alternative initiation codons usage and even ribosomal frameshifting. Until recently it was thought that eight genome segments encode at least 11 viral proteins (polymerase proteins PB1, PB2 and PA, nucleoprotein NP, hemagglutinin HA, neuraminidase NA, matrix proteins M1 and M2, non-structural proteins NS1 and NS2 (NEP). However at least four more non-structural proteins PB1-F2, PB1-N40, PA-X and M42 were found in recent years. Their role is probably associated with virus-host interactions.

It was also noticed that an additional ORF (called NEG8) in the positive orientation exists in NS gene segment of human IAVs, however the corresponding mRNA or protein have not been yet detected. Using RT-PCR and RNA hybridization techniques we showed the existence of non-genomic polyadenylated RNA (named NS3) corresponding to NEG8 ORF. It was detected in cells of different types, infected with human IAV strains. The mechanism of NS3 RNA formation is still to be determined. However, we’ve showed that IAVs segment 8 is able to encode the non-genomic negative sense RNA. Thus, IAV genome coding potential is not only limited by positive sense mRNAs, but it also can be ensured by negative sense RNAs, such as NS3.

SW01.S2–66
Expression of miR-29 in chronic myeloid leukemia patients after imatinib treatment
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Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm with an incidence of one-two cases per 100,000 adults and accounts for about 15% newly diagnosed cases of leukemia in adults. CML is characterized by a translocation between chromosomes 9 and 22, known as the Philadelphia chromosome. It leads to generation of a specific fusion protein – tyrosine kinase BCR/ABL. Tyrosine kinase inhibitor – Imatinib has been approved for the first-line treatment of patients with newly diagnosed CML. Successful treatment with imatinib is demonstrated by a significant decrease of BCR/ABL level in patient’s blood, reaching undetectable level in the course of several months [1].

Altered expression levels of microRNAs (miRNAs) have been described in many types of leukemias such as CML. miRNAs are small regulatory RNAs that affect protein expression post-transcriptionally, including suppressing translation or causing mRNA degradation. In this study we determined the expression level of miR-29, known as a tumour suppressor, because it modulates the expression of several proteins involved in apoptosis, including Bcl-1, Mcl-1. It was described that transfection of Philadelphia chromosome positive cells with miR-29b isoform causes apoptotic death of these cells [2].

Our results showed that the expression level of miR-29 is decreased in CML patients in comparison with control group and another hematopoietic disorder. Increased expression level of miR-29 was found after 2–3 months treatment with imatinib. Statistical analysis showed significant negative correlation between alterations of BCR/ABL and alterations of miR-29b and c. The two miR-29 isoforms are located on chromosome 1 and their expression is regulated by c-Myc and Smad3. Our data suggest that BCR/ABL affects expression of miR-29 and increasing level of miR-29b by other means may aid CML treatment.
SW01.S2–67
Optimization of the preparation of tissue material by using the method of laser microdissection for molecular studies
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A major difficulty in studies on biological material from tumors is obtaining a homogeneous cell population group due to heterogeneity of its cells. Laser microdissection (LM) is a modern technique that allows obtaining single cells or their homogeneous population out of heterogeneous material. Obtaining homogeneous populations of cancer cells from operating human material to study at the gene expression level requires special attention and care [1,2,3]. Scientific goal of my project is optimizing the procedure for preparation of tissue material for laser microdissection (LM) to obtain RNA of sufficient integrity and concentration from a homogeneous population of cells (obtained by LM), which will provide research of molecular biology in the future. The process of obtaining single cells or their homogeneous population out of heterogeneous material by using laser microdissection consists of several steps. It should be remembered that proper preparation of the starting material (collection and preservation) is a prerequisite for effective conduct of further molecular analysis. The ideal way of fixation of the biological material should protect both the cell morphology and the integrity of the noteworthy molecules. We assume that isolation of good quality RNA from cells obtained by LM constitutes an appropriate optimisation of techniques of preparation of materials for laser microdissection. Agilent 2100 bioanalyzer was also used, allowing us to measure both the concentration and ratio of RNA integrity — RIN (RNA Integrity Number), which will enable us to determine the quality of RNA obtained. Comparison of RIN values of RNA obtained from control samples (the starting material before proceeding to LM) and a pure population of cells (obtained by LM) allows to optimize the method of laser microdissection [1,2,3].

References

SW01.S2–68
FISH detection of single transcripts – the effects of improved probe design and advanced microscopy
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Due to recent, significant advances in techniques of visualization and detection of single molecules in eukaryotic cells precise localization and quantification of mRNA transcripts in situ is now possible with exceptional sensitivity and specificity. These techniques have significantly impacted the field of molecular and cell biology as well as medical research. We have adapted two different approaches in designing oligonucleotide probes for the fluorescent in situ hybridization (FISH) with single transcripts. The Stellaris probes are the sets of multiple, short oligonucleotides, each terminally labeled with a single fluorophore [1], while the Singer-type probe sets are composed of fewer and longer oligonucleotides, carrying internal fluorescent labels, typically at 4–5 positions [2]. We have performed FISH detection of mRNA transcripts for several genes expressed at various levels, in several types of cells (HeLa, lymphocytes, epithelial cells), in assays involving single and multiple probe sets. The visualization of fluorescent signals was performed using either fluorescent or confocal microscopy and several computational methods for signal enhancement and data interpretation were applied. Hereby, we present results of our tests and discuss advantages and drawbacks of evaluated methods with emphasis on their optimal configuration for detection of single molecule RNAs.

References

SW01.S2–69
HEN1-directed labeling of microRNAs
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Small non-coding RNAs are essential sequence specific negative regulators that can be found in such phylogenetically disparate groups as viruses and animals. One of their classes – microRNAs (18–25 nt), in most cases binds to 3' untranslated region of target mRNAs to repress their translation and/or stability. Since in animals this binding does not require full complementarity between microRNA and its target, one mRNA can be regulated by many microRNAs and vice versa. Indeed, more than 60% of human protein-coding genes are predicted to have microRNA binding sites within their 3' untranslated regions. Being one of the most abundant classes of gene-regulatory molecules microRNAs contribute to many biological processes in cells varying from housekeeping functions to responses to environmental stress. Like microRNAs are important in normal functioning of an organism so their mutations, dysfunctions and dysregulations are associated with many diseases including cancers, cardiovascular diseases, Alzheimer’s disease, schizophrenia, obesity etc. For fast and efficient disease diagnostic and appropriate treatment detection techniques for high-throughput microRNA profiling need to be developed. We present the novel small RNA labelling technology which recruits the HEN1 methyltransferase to attach the extended side chains
with functional groups towards 3'-end of microRNA. The method provides two strategies: quick and effortless one-step labelling through the direct attachment of the relevant reporter group which is embedded in the transferred radical and, alternatively, the two-step approach, which extends a choice of coupling strategies for manifold label conjugation to functional group.

SW01.S2–70
The transcriptionally- and translationally-acting ypaA riboswitch in Bacillus subtilis
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The 5' leader regions of many bacterial messenger RNAs contain regulatory elements - termed riboswitches - that able to form alternative secondary structures, depending on the presence of a specific ligand, and switch on or off the process of transcription elongation of mRNA or translation initiation of mRNA. The regulation of B. subtilis ypaA gene encoding a riboflavin transport protein is known to be under the control of flavin mononucleotide (FMN)-sensing riboswitch. In the current study we report an in vivo expression analysis of the effect of mutations in conserved bases located in the B. subtilis ypaA leader region. The wild-type and mutated ypaA leader regions were subcloned into plasmid pDG268 and pDG246 to generate, respectively, transcriptional and translational fusions to a lacZ reporter gene. All fusions were inserted into the chromosomal amyE locus of two isogenic B. subtilis strains: wild type and ribC1 mutant with reduced synthesis of endogenous FMN. Whereas the wild-type ypaA-lacZ fusion exhibited low expression in wild type strain and high expression in ribC1 background, the mutants tested displayed different phenotypes depending on the position of base substitution. To directly estimate the effect of FMN on the transcription of ypaA leader region, we carried out an in vitro transcription assay using purified RNAP in the absence or presence of 50 μM FMN. We found that FMN causes premature transcription termination at position +240 in the leader region of ypaA gene. A primer extension inhibition ('toeprint') assay was used to investigate the effect of FMN on binding of the 30S ribosomal subunit to the B. subtilis ypaA ribosome-binding site (RBS). It was shown that the abundance of extension products at the position 16 nt downstream of the AUG start codon decreased in trans (Suppl. 1) (2013) 3–617

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SW01.S2–72
Regulation of the rplY gene encoding 5S rRNA binding protein L25 in Escherichia coli and related bacteria
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Ribosomal protein (r-protein) L25 is one of the three r-proteins (L25, L5, L18) that interact with 5S rRNA in eubacteria. While L18 and L5 are universal r-proteins, L25 is typical of solely bacterial ribosomes as it is absent from archaean and eukaryotic translation apparatus. Although the rplY gene encoding L25 is not absolutely required for survival, E. coli cells deficient in L25 reveal a slow growth phenotype, indicating an important role of L25 in ribosome functioning. Specific interaction of L25 with a certain domain of 5S rRNA, a so-called loop E, is well studied by biochemical and physical techniques. At the same time, no information exists about regulation of L25 synthesis. While the rplR (L18) and rplE (L5) genes belong to the polycistronic sec-operon and, accordingly, their expression is regulated at the translational level by r-protein S8 that acts as an operon-specific autogenous repressor, the rplY gene represents an independent transcriptional unit, possessing its own promoter and terminator regions. The main goal of this work was to study the regulation of the rplY gene. We show here that the rplY promoter is highly conserved in a number of gammaproteobacterial families and is negatively controlled by ppGpp/DksA in response to amino acid starvation. The rplY 5'-untranslated region (5'-UTR) is also well conserved and bears some specific features, including a highly conserved stem-loop structure followed by an extended AU-rich single-stranded region. The most remarkable feature is the absence of the canonical Shine-Dalgarno element in front of the initiation codon. Despite this, the rplY-lacZ single-copy (chromosomal) reporter shows unusual high translation efficiency. This is reminiscent of the fs and rpsD 5'-UTRs which are also very efficient in translation despite the lack of the Shine-Dalgarno region. The synthesis of L25 in trans decreased translation
yield, indicating the mechanism of autogenous repression. Site-directed mutagenesis of the 5'-UTR structure revealed an important role of the conserved elements in the L25 autogenous control. Thus, the rplY gene expression regulation represents a novel example of the regulatory loops that are involved in the control of ribosome biogenesis in bacteria. (This work is supported by the RFBR grant 12-04-01138a).

**SW01.S2–73**

**Influence of let-7d and miR-18a overexpression on the radiosensitivity of hypopharynx squamous cell carcinoma**


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**Introduction:** Hypopharynx squamous cell carcinoma (HSCC) is one of the worst prognosis cancers of the upper aerodigestive tract and radiotherapy after surgery is the main method of treatment. miRNAs are good candidates for describing radiosensitivity of tumors what consequently may lead to personalization of radiotherapy. Let-7d acts as tumor suppressor of C-MYC and miR-18a acts as suppressor of K-RAS, but the role of these miRNAs are still controversial and unclear. Our previous data showed correlation between these two miRNAs. That observation led us to explain this phenomenon and its influence on cells behaviour after irradiation.

**Purpose:** Influence of overexpression of let-7d, miR-18a and both of them on radiosensitivity of FaDu cell line (hypopharynx squamous cell carcinoma).

**Methods:** Different overexpression of let-7d and let-7d-miR-18a were achieved using lentiviral system. Expression level of miRNAs were estimated by qRT-PCR method. The proliferation ratio of cell models was analyzed by MAT assay. FaDu-7d and FaDu-7d-miR18a cell models were irradiated using dose of 2 Gy in water phantom with Clinac 2300 followed by clonogenic assays. FaDu-GFP and non-modified FaDu cell models were used as controls. miR-18a cell models are now under construction.

**Conclusion:** We received FaDu-7d and FaDu-7d-miR18a models with different overexpression range from 10- to 43.5 times higher than in non-modified cells. Overexpression of let-7d does not significantly change expression of miR18a in modified FaDu-7d cell lines. The cell models have different proliferation ratio depending on expression level of let-7d and miR18a. Surprisingly, cell lines with double overexpression of miRNA let-7d and miR-18a simultaneously have higher proliferation rate than in single miRNAs' overexpressing. The clonogenic assay showed different proliferation ability and radiosensitivity of FaDu-7d and FaDu-7d-miR18a models compared to controls and among themselves.

**Conclusions:** Let-7d shows double nature according to its expression level in cell. Let-7d may act as tumor suppressor when overexpression is 10 or 21 times higher than in control cell line and as oncorm when overexpression level is 16- and 43 times higher. This phenomenon we will explain by analysis of differences in total expression pattern of each modified cell lines.

**SW01.S2–74**

**Analysis of microRNAs expression changes after irradiation of oral and tongue squamous cell carcinoma lines**


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**Introduction:** The aim of the study is an identification of CD44+/CD133+ cell population, known in HNCs as cancer stem cells, and characterize its microRNA profile. It is believed cancer stem cells are responsible for cancer progression, chemo- and radioresistance of tumor. In the first step, we analysed expression profile of selected miRNAs of total cell population after irradiation.

**Purpose:** (i) Analysis of changes in expression levels of miRNAs after irradiation, (ii) characterization of CD44+/CD133+ cell population.

**Method:** Changes of miRNAs expression were marked by qRT-PCR in SCC-25 (tongue) and SCC-040 (oral) cell lines after 2 Gy irradiation in water phantom with Clinac 2300. CD44+/CD133+ population of each cell line was analysed by flow cytometry.

**Results:** Expression of selected miRNAs in irradiated cells was compared with expression of miRNAs from non-irradiated cells. We observed differences in miRNAs expression profile between SCC-25 and SCC-040 cell lines. All of examined miRNAs were up regulated between the 1.5 and 48 h after irradiation in both cell lines. Up regulation of miRNAs was dynamic process with two highest picks between the 1.5–2.5 and the 6–12 h. The lowest level of miRNAs expression was observed at 4.5 h. In both cell lines, almost total population of cells was CD44+ positive. CD44+ cells expressed higher CD133 level than CD44- cells. We observed 2.3% and 0.6% of CD44+/CD133- cells in whole population of SCC-25 and SCC-040. However, reduction of FBS concentration caused significant increase of CD44+/CD133- population in both cell lines.

**Conclusion:** (i) Analysed miRNAs are up regulated in the highest level in two time points after irradiation, (ii) differences in genomes of examined cell lines may result in characteristic response to irradiation showed in similar, but not the same, expression of miRNAs, (iii) examined miRNAs profile seems to be useful tool for characteristic of CD44+/CD133+ cell population, and may influence on treatment effectiveness.
sequence stimulating translation termination. In this study both approaches were applied. Search of the most rare and frequent stop codon contexts in human genome was carried out and estimation of their participation in translation termination was experimentally defined. For this purpose completely reconstructed \textit{in vitro} eukaryotic translation system was used. As a result we have found out dependence of the translation termination efficiency on different stop codons from A/G composition of 3' contexts. For UAA stop codon the most efficient and frequent contexts are A-rich, for UAG and UGA – G-rich.

**SW01.S2–76**

Glycyl-tRNA synthetase modulates eIF4G activity to promote correct initiation of translation on type I picornavirus IRESes

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Glycyl-tRNA synthetase serves as unusual IRES transacting factor for type I picornavirus IRESes, as its recruitment to tRNA anticodon-like element located in domain V is necessary for efficient translation initiation. Here we show that in RRL, which is deficient on GARS, the translation driven by PV and HRV IRESs can be stimulated by addition of either p100 fragment of eIF4G or by smaller 4G middle domain. However, for RRL supplemented with GARS, the effects of p100 and 4G MD on translation becomes opposing: whereas p100 still stimulates translation, 4G MD inhibits it. Intriguingly, when putative upstream initiation codon AUG\textsubscript{SS} in PV IRES is mutated, the stimulatory effect of GARS on translation is reduced and 4G MD effect is reversed from inhibition to stimulation. Consequently, when repressor gene is translated directly from AUG\textsubscript{SS}, the effect of GARS on translation becomes more prominent and 4G MD starts to act as inhibitor. We propose that GARS stimulates eIF4G binding to IRESs of type I and regulates ribosomal entry to putative upstream AUG from which it is redirected to correct initiation codon.

**SW01.S2–77**

The mechanism of transcription antitermination by the p7 protein of \textit{Xanthomonas oryzae} phage Xp10

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Xp10 is a lytic bacteriophage of the \textit{Siphoviridae} family that infects \textit{Xanthomonas oryzae}, a plant pathogen that causes bacterial leaf blight disease in rice. P7 is a small protein of Xp10 that cooperates with host RNA polymerase (RNAP) from lysates of infected bacterial cells. Previously, it was shown that p7 affects various stages of transcription by the host RNAP. In particular, p7 inhibits promoter recognition on a subset of bacterial promoters and suppresses intrinsic termination of RNA synthesis. However, the mechanism of p7-mediated antitermination remains unclear. In our work, by using purified RNAP and transcription factors from \textit{X. oryzae}, we found that the p7-mediated antitermination depends on the flap domain of the \(\beta\) subunit of host RNAP and is enhanced by cell-encoded protein NusA. In this respect, p7 acts similarly to the N and Q antiterminator proteins of phage \(\lambda\) that also interact with the \(\beta\) flap domain and depend on NusA for their action. P7 also stimulates transcription elongation by RNAP and suppresses certain classes of transcription pauses. The results of analysis of transcription antitermination in artificially assembled transcription complexes suggested that p7 likely acts by suppressing transcription pausing and stabilizing transcription elongation complexes. Analysis of the roles of p7 in phage development and cell infection will be an important goal of future studies.

**SW01.S2–78**

Endonuclease cleavage is the first event of human telomerase RNA 3'-end processing

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Human telomerase RNA (hTR) is synthesized by RNA polymerase II from its own promoter as a long precursor. Multiple forms of telomerase RNA that differ at 3'-end were identified but precise position of the transcription stop of pre-hTR remains unknown. The mature form of hTR is produced by 3'-end truncation and 5'-end modification with a trimethylguanosine (TMG) cap. Expression of polyadenylated hTR-transcript from CMV-, CAG- and PGK-promoters led to accumulation of unprocessed form of hTR. The insertion of U1-terminator just after hTR also inhibited hTR processing. hTR containing various sequences at 3'-end of hTR expressed from polymerase II and polymerase III promoters were processed to different extend. These data and recent investigation of the role of H/ACA domain in telomerase RNA maturation indicate that formation of RNP complex at H/ACA domain is crucial for correct hTR processing. However, the question remains whether hTR maturation involves endonuclease cleavage of primary transcript or it is processed by 3'-end exonucleolytic trimming as in classical snoRNAs.

To discriminate between these possibilities we have decided to exploit stable cell lines in which RNA from the construction containing the sequence of mature hTR (451 nt) with the flanking 424 nt of the 3'-genomic sequence followed by fluorescent protein coding region with IRES-element is expressed from polymerase II promoter. Our data suggest that hTR 3'-end processing is initiated by endonuclease cleavage.

**SW01.S2–79**

Energetics of translocating ribosome

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Every elongation cycle of protein synthesis is completed with the translocation step, a synchronous movement of the mRNA by one codon, together with the movement of deacylated tRNA from the P to the E site and the peptidyl-tRNA from the A to the P site. In bacteria, rapid translocation is catalyzed by the elongation factor G (EF-G) at the cost of GTP hydrolysis, thus contributing to the overall high rate of protein biosynthesis. Recent data indicate that for some tRNAs translocation can proceed in reverse direction [1]. In the absence of EF-G and GTP hydrolysis, the direction of translocation is determined by the thermodynamic gradient formed by the different affinities of tRNA binding to the A, P, and E sites. Structural and biochemical studies of non-catalyzed reverse translocation has demonstrated the coupling of tRNA movement with conformational changes of the ribosome [2], implying that translocation is an intrinsic function of ribosome-tRNA complex. Studies of interac-
tion of misacylated peptidyl-tRNA with the ribosome in pre- and post-translocation states revealed that the tRNA moiety rather than the amino acid determines the propensity of valine-specific tRNA for efficient reverse translocation. Upon the peptidyl transferase reaction peptidyl-tRNA bound to the A site [3] and deacylated tRNA bound to the P site are destabilized, thus changing the thermodynamic profile of ribosome for the following reaction of translocation. Binding of EF-G (in GTP-mode) to the ribosome makes the thermodynamic profile favorable for the forward translocation, and energy of GTP hydrolysis is utilized to accelerate formation of the ‘unlocked’ conformation of the ribosome and to allow for uniformly rapid and complete translocation despite of the properties of particular tRNAs.

References

SW01.S2–80
Does HIV-1 mRNA leader possess an IRES?
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Despite many efforts, mechanism of HIV-1 mRNAs translation still remains obscure. Conventional wisdom says that it can use both scanning mechanism and internal initiation, the latter prevailing in G2/M-phase. However, since existing reports are equivocal, we performed extensive analysis of the unspliced HIV-1 mRNA 5'UTR using stringent criteria for an IRES identification, that have been recently developed in our lab.

Our results strongly point to the lack of any IRES in HIV-1 mRNA leader. First, direct comparison of translation efficiencies of monocistronic versus bicistronic mRNAs revealed that HIV-1 mRNA 5'UTR promotes internal initiation rather poorly both in vitro and in vivo. In addition, monocistronic mRNA translation was strongly stimulated by capping and, moreover, translation of both capped and uncapped mRNAs was severely inhibited by introduction of upstream AUG-codons to a similar extent. These results were reproduced in several unsynchronized cell lines, including Jurkat cells, as well as in G2/M-starved ones.

Overall, we conclude that HIV-1 mRNA is translated exclusively in a cap-dependent fashion.

SW01.S2–81
Alpha7 subunit of proteasomes participates in gene expression regulation
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Proteasomes are ATP-dependent proteases composed of 20S core particle and two associated with 19S regulatory complexes. Core consists of four rings, each being formed by subunits of alpha- or beta-type. Besides well-known ubiquitin-dependent mechanism of protein degradation, there is ubiquitin-independent protein degradation that is also performed by proteasomes. Some of these proteins are known to interact with 20S core subunit alpha7.

In this study we evaluated RNase activity of proteasome subunits and determined proteins that can bind alpha7.

Alpha7 obtained with RT-PCR was cloned into pGEX-5X-3 expression vector, recombinant GST-alpha7 protein was expressed in E. coli and purified by affinity chromatography. In order to identify the proteins that can interact with alpha7 we prepared cytoplasmic and nuclear extracts form human K562 cell. Extracts were incubated with GST-alpha7 or GST bound to glutathione beads. Proteins bound to GST-alpha7 or GST were separated by 2D electrophoresis and analyzed by mass-spectrometry. We were able to identify 62 proteins interacting with GST-alpha7 but not to GST, including proteins responsible for transcription and translation.

Since alpha1 and alpha5 subunits of 20S proteasome were shown to have RNase activity it was important to check whether other alpha-type subunits, and alpha7 in particular, possess this activity as well. p53 mRNA and 3'-untranslated region of c-myc mRNA were used as substrates, recombinant GST was used as negative control and purified alpha5 as positive control. RNA degradation was analyzed by PAGE. Recombinant alpha7 was shown to have RNase activity comparable to those for alpha5.

Taken together, our results demonstrating the RNase activity of alpha7 in vitro and showing the interaction between alpha7 and various proteins involved in transcription and translation, suggest a possible role for proteasomes in many levels of gene expression.

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SW01.S2–82
The dual functional role of Mir-9 in regulating migration and growth of glioma cells
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Migration-proliferation dichotomy is a common phenomenon in gliomagenesis; however, the exact molecular mechanism of this ‘go or grow’ phenomenon remains largely incomplete. In our study, we found a proliferation-inhibitory role for miR-9 by targeting cyclic AMP response element-binding protein (CREB) and neurofibromin 1 (NF1), in glioma cells, which appears inconsistent with its high expression level. We also confirm a migration-enhancing role of miR-9, suggesting dual roles for miR-9 in modulating the proliferation and migration of glioma cells. Interestingly, the expression of NF1 is positively regulated by CREB. Our study proposes a potential negative feedback minicircuitry comprising CREB and miR-9, where CREB contributes to the transcriptional activation of miR-9-1 and miR-9 represses the expression of CREB at the post-transcriptional level. Importantly, our study suggests that the balance between CREB and miR-9 determines the ‘go or grow’ status of glioma cells, furthering our understanding of the transition from proliferative to migratory phenotype during glioma progression.
SW01.S2–83
Telomere repeat transcription in chicken growing oocytes: new data gained with cytological approach
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Transcripts of C-strand of the telomere repeat were revealed at the ends of avian lampbrush chromosomes almost 20 years ago (Solovei et al., 1994). More than a decade later, interest to the phenomena of telomere transcription had revived with the discovering of telomere repeat containing RNA (TERRA) in mammalian cells (Azzalin et al., 2007). Great progress has been made in exploration into TERRA-RNA biogenesis. It was shown that TERRA-RNA is transcribed by RNA-polymerase II and acquire 5′-7-methylguanosine cap. Major fraction of mammalian TERRA-RNA is associated with telomere chromatin and do not contain poly(A) tail, whereas minor nucleoplasmic fraction of TERRA is polyadenylated (Porro et al., 2010).

Usage of lampbrush chromosomes (LBCs) allows to co-localize various transcripts with RNA-polymerases and/or processing factors at the level of individual transcription units. RNP-structures containing G-rich transcripts of telomere repeat terminate all chicken LBCs (Solovei et al., 1994). In this study we questioned whether telomere repeat transcripts (TelRNA) on chicken LBCs represent nascent transcripts. To answer this question we localized TelRNA using RNA-FISH with Cy3 labeled (CCCTAA)5 oligonucleotide probe after immunofluorescent detection of phospho-CTD of RNA-polymerase II or BrUPT injected into oocyte. Fluorescent signals indicating injected BrUPT did not co-localized with RNA-FISH signals and transcribed chromatin axis of the terminal lampbrush loop ended at the base of RNP-structure containing TelRNA. This suggests non-nascent nature of the TelRNA, and if so, TelRNA should have been 3′-cleaved and perhaps polyadenylated. RNA-FISH with oligo(dT) 30-bio probe and reverse transcription in situ revealed poly (A) + RNA within terminal RNP-structures on chicken LBCs. After two-color RNA-FISH fluorescent signals from TelRNA and poly (A)+ RNA did not overlapped, and the latter always had more distal position. Our data suggest that terminal RNP-structures on chicken LBCs accumulates non-nascent telomere repeat transcripts, which might be polyadenylated.

SW01.S2–84
Posttranslational modification of protein S6 in E. coli leads to suppression of translation in stationary phase
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Ribosomal protein S6 in Escherichia coli has a unique type of posttranslational modification, addition of extra amino acid residues to C-terminus. Two C-terminal glutamate residues of protein S6 are encoded in genome, while specific enzyme RimK adds up to four extra glutamate residues, forming heterogenic acid tag. We show that modification takes place in stationary phase; S6 is not modified in logarithmic phase.

For determination of the functional role of this modification we used two E. coli K-12 strains, wild type (WT) and ΔrimK. Comparison of the native proteome of WT and ΔrimK cells shows that total protein synthesis in WT cells in stationary phase is reduced much more then that in ΔrimK cells. However the total protein synthesis in logarithmic phase is equal in these two strains. This result suggests that modification of protein S6 in stationary phase leads to suppression of translation.

SW01.S2–85
Does the component of telomerase complex Est3p interact with telomeric quadruplexes in yeast?
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Telomerase is a key participant of telomere length maintaining system in the majority of eukaryotes. It synthesizes telomere repeats at the 3′-end of telomeric DNA using its own RNA template. The Saccharomyces cerevisiae telomerase contains besides the reverse transcriptase subunit and RNA subunit Est1, which is necessary for telomerase attachment to telomeres and telomerase activation, and Est3 with an unknown function. It is also believed that Est1 is able to catalyze G-quadruplex formation from telomeric repeats. In the same time Est3 is predicted to have an OB-fold. Its ability to interact with single strand telomeric DNA has long been discussed in the literature. We have tested the ability of Est3 from two organisms (S. cerevisiae and H. polymorpha) to interact with telomeric quadruplexes and shown serious differences in the properties of these proteins.

SW01.S2–86
The role of short trans sense-antisense interactions in regulation of gene expression using TurboGFP as a model gene
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RNA-RNA interactions are known to play a significant role in regulation of gene expression. Natural antisense transcripts (cis-NATs and trans-NATs) are RNAs that contain sequences complementary to other endogenous RNAs. cis-NATs have been studied intensively and found to participate in regulation of gene expression while studies of trans-NATs have generally focused on small RNAs – siRNAs and miRNAs. Recently, short trans sense-antisense pairs (15–25 bp length of overlapping) between human protein coding transcripts have been discovered using bioinformatical approach and it has been indicated that such mRNA-mRNA pairs are more common than expected by chance. Our study focuses on investigation of involvement of short trans sense-antisense interactions occurring between two mRNAs in regulation of gene expression using experimental approach. TurboGFP gene was used as a model gene. Based on RefSeq database we identified genes which had different length of overlapping (16 bp) with TurboGFP mRNA. TurboGFP was introduced in different human cell lines and RNA isolated from these cell lines was used in gene expression analysis. Real-time PCR showed that TurboGFP expression influenced on mRNA level of genes with different length of overlapping even with minimal length (16 bp). Using RNA interference we confirmed that differences in gene expression profiling between TurboGFP-positive and Turbo-GFP-negative cells really depended on the level of TurboGFP mRNA. This study contains first evidences of the role of short trans sense-antisense interactions in regulation of gene expression.

SW01.S2–87
Study of the structure and function of the novel gene asASCL1 in human
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Examination of the phenomenon of antisense regulation of gene expression is urgent as recent years it has been demonstrated that
some natural antisense transcripts (NATs) play an important role in sense genes expression including those which are involved in pathogenesis of a number of diseases. However, the functions and the mechanism of antisense regulation are not enough studied nowadays.

Lately in our laboratory in silico search of overlapping clusters of EST relating to opposite DNA strands has been conducted. We chose a cluster with a gene ASCL1 among the obtained cases. Protein ASCL1 belongs to a family of transcription factors, which contain bHLH (basic helix-loop-helix)-domain responsible for DNA binding. It was found that it played a role in the neuronal commitment and differentiation. Also this gene possible plays role in contributing to progression of tumors.

Based on the analysis of EST obtained from the GenBank database we supposed the presence of NAT to ASCL1 gene. This hypothesis was proved experimentally using quantitative PCR, Northern blot analysis and strand-specific RT. Then we identified 3′- and 5′-ends of this transcript using RACE-PCR. As a result, we have described novel gene in human – asASCL1, which is transcribed from the opposite strand to ASCL1 sense gene, and overlaps with its counterpart in conservative bHLH-region. We also hypothesized that transcript of asASCL1 gene could participate in antisense regulation of expression level of ASCL1-gene.

The further in silico analysis indicated that human ASCL1 gene is part of the ASCL-gene family, which also includes genes: ASCL2, ASCL3, ASCL4 and ASCL5. We identified that mRNA sequences of this genes had significant regions with high homology which were overlapped with the asASCL1 transcript. On the basis of these facts we made a hypothesis that a whole gene family could be trans-antisense regulated by one antisense transcript with different efficiency for each of its members depending on homology level of an antisense transcript and mRNA sequence of genes.

**SW01.S2–88**

**A new method for m6A identification in RNA**

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Modified nucleotides in RNA are widely-spread among all organisms. One of the most frequent modification is methylation. The role of the modification process can be very diverse depending on the RNA type, its structure and function. The greatest interest lies for us in understanding of the significance of m6A modified nucleotide for RNA functioning. The main problem in our research comes up during the stage when RNA carrying a concrete m6A modification and unmodified RNA should be differentiated one from another. Large majority of existing methods allows us to determine the presence and in some cases – to estimate the number of modified nucleotides. But it’s still difficult to find cheap and simple method for determination of the occurrence of concrete m6A modification in the required RNA sequence.

Here we present a new method for m6A-detection in RNA by means of melting of RNA-DNA duplexes formed by studied RNA and two oligodeoxyribonucleotides with different length that are both complementary to RNA in area of its concrete modified nucleotide. In our system the shorter oligonucleotide is modified by the Black Hole Quencher (BHQ1) at the 3′-terminus and hybridizes accurately with the nucleotide of interest and some neighboring nucleotides of RNA. The longer oligonucleotide is modified by FAM at the 5′-terminus and hybridizes with the RNA just near the shorter one. To determine the existence of modified nucleotide in studied RNA, a control RNA with known modification status should be chosen.

The identification is accomplished by comparing melting curves for control RNA and RNA under study.

We showed that the correct pair of such oligonucleotides allows us to get a remarkable difference in differential melting curves between methylated and non-methylated nucleotide in rRNA.

**SW01.S2–89**

**miRNA implication in the most common subtypes of renal cell carcinoma and urothelial carcinoma of the upper urinary tract**

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The most common renal cell carcinoma (RCC) subtypes are ccRCC, papRCC and chRCC. Upper urinary tract-urothelial cell carcinomas (UUT-UC) account for only 5–10% of urothelial carcinomas. MicroRNAs (miRNAs) are small non-coding RNAs, recently found to be deregulated in RCC. We identified miRNA signatures that can distinguish RCC subtypes, accurately. Furthermore we focused on biomarker discovery, identification of gene targets and consequences of miRNA deregulation. Twenty-seven FFPE RCCs and UUT-UCs were profiled. Results of significantly deregulated (DE) miRNAs were validated using qRT-PCR and LNA-ISH. The ability to discriminate between the RCC subtypes and normal tissue was characterized by ROC. Chromosomal distribution of the DE miRNAs was compared with reported genomic alterations. MiRNA target prediction was performed by miRWalk. Enriched gene sets were grouped in functional categories by IPA and GO enrichments. The majority of the miRNAs (69.8%) was down-regulated in RCC. Unsupervised hierarchical clustering with Euclidian distance successfully managed to classify the various RCC subtypes among them. Microarray and qRT-PCR results revealed similar expression patterns, qRT-PCR validated the expression of miR-3648, miR-489, miR-638, miR-3656, miR-3687, miR-663b, miR-25-5p and miR-21-5p in ccRCC. MiR-25-5p high expression was confirmed in all ccRCC, papRCC and chRCC sections by LNA-ISH and its expression was significantly stronger compared to their corresponding normal tissues. More aggressive ccRCCs also stained stronger than the less aggressive ones. Chromosomal distribution analysis revealed that, for each RCC subtype, miRNAs had deregulated patterns that agreed with some of the previously reported chromosomal gains and losses. Four major gene networks were constructed by IPA for the DE miRNA targets. MiRNAs are deregulated in RCC and may contribute to kidney cancer pathogenesis by targeting key molecules involved in tissue development, cell cycle and proliferation.

**SW01.S2–90**

**Investigation of applicability of plant extracts (Euphorbia orientalis L.) instead of chemical disinfectants**

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Euphorbiaceae, the Spurge family is a large family of flowering plants with 300 genera and around 7500 species. Euphorbia genus
have been investigated for a long time in view of different specialties, like more energy content as alternative source of hydrocarbons, laticifiers, phytochemicals and systematic. Mediterranean Euphorbia species have been the object of various studies and they have been proposed as potential renewable sources of unsaturated. Negative impact on the environment shows that the use of chemical disinfectants. Therefore, in our study, disinfector effects of plant extract (Euphorbia orientalis L.) were investigated that found as free in nature. Antimicrobial and antioxidant activities determinate by using leave samples of Euphorbia orientalis L. collected from Corum/Turkey and environment in this study. Chloroform and ethanol extracts of plant were examined for antioxidant and antimicrobial activities. Antioxidant activities were detected spectrophotometrically by using Erel (2004) methods. Antimicrobial activities of Staphylococcus aureus ATCC 29253, Candida albicans ATCC 10231, Escherichia coli ATCC 25922, Enterococcus faecalis ATCC 29212 and Pseudomonas aeruginosa ATCC 27853 strains evaluated by disc diffusion method. And also, GS/MS analyses were carried out by chromatographic. On all microorganisms of plant extracts observed inhibition effects in different rates. The most sensitive Candida albicans strain (15.0 ± 1.0 mm) and also the most resistance strain Staphylococcus aureus (10.0 ± 1.0 mm) were determinate in antimicrobial activity studies. An antioxidant activity of plant has observed in our studies. And also, antioxidant activities of ethanol extract were higher than chloroform extract. At the same time, diphenic acid derivatives were found nearly the content of all extracts.

**SW01.S2–91**
The peculiar mode of translation elongation inhibition by antitumor drug harringtonin

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Plant alkaloid harringtonin (HT) was isolated in the 1960s from the bark of evergreen plant Cephalexous harringtonia grown in China. It belongs to the group of cephaloxine derivatives, complex heterocyclic compounds having a strong anti-tumor effect. HT inhibits protein synthesis in eukaryotic cells. It is known from *in vitro* experiments that HT acts at the stage of elongation by binding to the A-site of the large ribosomal subunit and interfering with peptide transferase reaction. However, when added to cells, HT causes rapid polysome disassembly, because HT traps only *de novo* formed 80S complexes and is unable to bind to those ribosomes that are involved in productive synthesis of a polyepitide within the polysome. Thus, HT addition should lead to accumulation of arrested elongation complexes at the very beginning of coding region, presumably at the first (start) codon of the mRNA. Although this hypothesis has never been verified directly, this feature of HT was used recently in ribosome profiling experiments to map mRNA start codons in a genome-wide scale.

We investigated the effect of HT on the elongation in a cell-free translation system using toe-printing technique. We found that HT poorly blocked elongation at the start codon of the beta-globin mRNA, but instead produced a very strong signal at the 9th codon encoded for Lys. Using a set of artificial mRNA constructs, we showed that HT specifically stalled elongating ribosomes in positions where their P-sites were occupied by either Lys, Arg or Tyr codons, while almost did not interfere with other amino acids. No influence of surrounding nucleotide or amino acid sequence, as well as no difference between synonymous codons, were detected. The same results were obtained with another cephaloxine derivative, homoharringtonin.

We assume that the amino acid specificity is dictated by a distinct architecture of the PTC occupied by peptideyl-tRNAs having either Lys, Arg or Tyr residues at the C-terminus of the nascent peptide. In this environment, HT either freezes the complex at pre-translocation step or blocks squeezing of the peptide chain into the ribosomal tunnel. These data reveal a rare example of an amino acid specific inhibition of elongation and extend our knowledge about mechanisms of action of PTC inhibitors.

**SW01.S2–92**
Inflammation-caused shifts in microRNA expression as trigger of tumor growth

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**Background:** Although inflammation is associated with tumor growth, molecular basis of this phenomenon remains unclear, especially when pathogens do not have the ability to damage DNA immediately. However, the inflammation entails regular changes in the expression of cell microRNAs (miRNAs). Expression of miRNAs miR-155, miR-21, miR-146a, miR-125b, miR-31, miR-34c, miR-200, miR-203 and miR-205 is usually up-regulated whereas expression of miRNAs miR-7a/b, miR-34a, miR-143, miR-145, miR-320a, miR-375, miR-379 and miR-434-3p is down-regulated. This investigation aims to identify in what way these shifts in miRNA expression pattern contribute to the cell transformation and tumor growth.

**Methods:** miRNA targets within gene transcripts were predicted in *silico* using TargetScan software.

**Results:** miRNA miR-143 can silence abl-2, bel-2 and *erbB3* genes, miR-145 targets *E2F3, RASAI/2*, CDK6, *erbB3*, B4, ESR1, *ACTB* and *ACTG1* genes. miR-320 suppresses *E2F1/3/7, RASAI, CDK6, p57, ESR1* and *ITG5* genes. Down-regulation of these miRNAs causes derepression of genes encoding key elements of proliferative and antiapoptotic signal pathways as well as genes responsible for cell motility and abnormal adhesion. Up-regulated miRNA miR-155 silences *CLDN1, CGN, OCLN, F11R (JAM-A), TGFBR2* and *SIRT1* genes and genes coding *alpha*-actinins. miR-21 can target *CLDN1, CGN, CADMI, VCL* and *TGFBR2* genes.

**Conclusion:** Inflammation is associated with miRNA expression shifts that lead to increasing of cell proliferation and survival as well as to silencing of antiproliferative and proapoptotic genes. Also, up-regulated miRNAs suppress genes encoding components of cytoskeleton and intercellular junctions. This results in alterations in cell-cell adhesion, impairs contact inhibition, facilitates cell motility and migration. Furthermore, miRNA miR-155 silences SIRT1 gene encoding the histone deacetylase sirtuin 1. This causes increasing of overall level of chromatin acetylation and expression and, therefore, makes possible the reactivation of silent oncogenes as well as transposons, which can rapidly lead to dramatic increase of DNA damage level and genome destabilization. Thus, inflammation creates epigenetic background for cell transformation as well as tumor promotion and metastatic spread.

**SW01.S2–93**
Human telomerase RNA 3′-end processing

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Telomerase is the essential enzyme that maintains telomere length in eukaryotes. Telomeres are essential for genome stability, protecting natural chromosome ends against fusion, recombination and degradation. The telomerase ribonucleoprotein is composed of two core components, telomerase RNA (hTR in humans) and reverse transcriptase protein subunit (hTERT in humans). The study of the processing of human telomerase RNA is complicated
by its low content in cells. We created stable cell lines with over-expression of the major components of telomerase. The expression level of hTR and hTERT mRNA was analysed by qRT-PCR. The level of hTR increased in presence of hTERT. It allows to propose the reverse transcriptase is necessary for hTR stabilization. Also we created the stable cell line with overexpression mutant telomerase RNA that had a deletion of two nucleotides in 3’-end sequence. Our data show that mutant form of telomerase RNA is processed better, than wild-type hTR.

**SW01.S2–94**

**De-novo transcriptome assembly and differential expression analysis of starfish, Asterias rubens**

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The Common Starfish or Asterias rubens (Echinodermata, Asteroidea) is the most common and familiar starfish found in Northern Atlantic region. Sea stars are using as a model organism for histological and cytological studies. Transcriptomic and genomic data for Asterias rubens are not published yet. In this work we performed de novo assembly of transcriptomic sequencing data of digestive caeca of Asterias rubens in normal and stress (influence 30°C during 30 min) conditions. All data were produced by next-generation sequencing using Illumina GAIIx (Institute of Molecular Biology RAS). A total of 64353659 and 55785208 76 nt paired-end reads were obtained from high-throughput sequencing for normal (1) and stress (2) conditions, respectively. In the absence of a genomic database, the correct de novo assembly of RNA-Seq is the rather difficult task. We have performed de novo assembly using Trinity [1] de novo transcriptome assembler. The total lengths of transcripts varied depending on the read preprocessing strategies and were 38498711–51605511 bp (1), and 52249984–57267908 bp (2). The total number of transcripts varied from 70444 to 83053 for (1) and from 84460 to 88577 for (2). The max length of transcripts varied from 13943 to 14100 bp for (1) and from 15284 to 14385 bp for (2). To discriminate differences between transcriptomes assembled from samples under normal and stress conditions and identify reference genes the differential expression analysis was performed.

I would thank to Anna Kudryavtseva, Nataliya Melnikova, and Anna Speranskaya from Engelhardt Institute of Molecular Biology for providing the data of next-generation sequencing (Illumina GAIIx).

**Reference**


**SW01.S2–95**

**Nuclear bodies involved in U snRNPs biogenesis in late stage pigeon oocyte nucleus**

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During spliceosomal U snRNPs biogenesis newly assembled U snRNPs reimport from the cytoplasm into the nucleus. In the nucleus, imported TMG-capped U snRNAs accumulate in nuclear bodies for final steps of their maturation before entering the speckles. There are several types of nuclear bodies enriched with U snRNPs: universal Cajal bodies and histone locus bodies, as well as coilin-positive spheres in pigeon late stage oocytes and pearls in oocytes of Xenopus. The Cajal bodies are most widely studied intranuclear domains enriched with components for U snRNPs modifications (methylation and pseudo ubiquitination). Nevertheless it was shown, that U snRNPs modifications also take place in coilin mutants without canonical Cajal bodies. The histone locus bodies contain factors required for histone pre-mRNA maturation. As we have shown previously, pigeon early stage oocytes contain these universal nuclear bodies: equivalents of Cajal bodies (enriched with coilin and gemini2), equivalents of histone locus bodies (enriched with symplekin) and nucleolus. At the same time until recently, there were no coilin-positive bodies described in avian late stage oocytes. Newly described coilin-positive spheres in pigeon late stage oocyte nucleus accumulate TMG-capped U snRNAs and associated Sm-proteins and apparently take part in U snRNPs biogenesis. These bodies do not accumulate symplekin or U7 snRNA involved into 3’-end processing of histone pre-mRNA. At the same time we demonstrate that these spheres lack gemini2 and fibrillarin, proteins being typical for Cajal bodies. The importance of the spheres during pigeon oogenesis remains unclear as there is no any coilin-positive spheres in late stage oocyte nucleus of other studied species of birds. On the one hand it seems that the spheres in pigeon late stage oocytes store some components required for further embryogenesis. On the other hand these coilin-positive domains might reflect mechanism of self-organization of conservative U snRNPs biogenesis machinery. Further experiments need to be done to reveal the nature of these remarkable nuclear structures.

**SW01.S2–96**

**Identification of molecular targets of RNase A antitumor therapy**

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In recent years, ribonucleases are regarded as perspective anticancer drugs. Novel data, suggesting an important role of miRNAs in mediating tumor growth and invasion, have provided researchers with a new area to search for possible molecular ribonuclease targets. Previously, antitumor and antimetastatic properties of pancreatic ribonuclease RNase A have been verified (Patutina O.A. et al., Biochimie 2011). The use of RNase A as an adjuvant in conjunction with conventional cytostatic cyclophosphamide resulted in a reliable enhancement of antitumor and antimetastatic effect of the therapy. In the present study, by high-throughput SOLID sequencing technology we performed analysis of genome-wide profiles of miRNAs in tumor and serum of mice after the treatment with RNase A. Sequencing data revealed that RNase A treatment resulted in an alteration in the levels of serum and tumor-derived miRNAs. RNase-mediated downregulation of miR-10b, miR-145a, let7 g and mir-451a was validated by qRT-PCR. The drop of miRNA expression was accompanied by upregulation of tumor-suppressive genes Pten, Timp3, CD82 and Brms1. The obtained data give the evidence that antitumor and antimetastatic effects of RNase A is associated with alteration in miRNA profiles in tumor tissue and blood serum promoting the strengthening of adhesive properties of tumor cells and impeding an ability to metastasize.

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SW01.S2–97
Stereochemistry of interaction between phosphorothioates at the guide strand of siRNA duplex and Ago2 protein
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P-chiral, stereodefined oligonucleotide analogs, in which one of a non-bridging phosphate oxygen atoms is substituted with another heteroatom (e.g. sulphur) or chemical group, offer possibilities for detailed studies of interactions with other biomolecules at the molecular level. Such probes are often used for elucidation the mode of action of various enzymes, for studies on the thermodynamic stability of nucleic acids complexes as well as they are very useful for analysis of interactions of the phosphoryl oxygen atoms in natural precursors with functional groups of proteins.[1].

Chemically synthesized small interfering RNAs (siRNAs) mediate efficient gene silencing in a sequence-specific manner by utilizing an endogenous RNA interference (RNAi) pathway. Argonaute 2 (Ago 2) protein binds siRNA duplex and uses the ‘guide’ strand sequence information to locate and silence complementary target mRNA by direct cleavage via the endonucleolytic ‘slicing’ reaction. A variety of chemical modifications have been evaluated for improving the potency, specificity and stability of siRNAs. Recently, we published the results of our studies on the properties of siRNAs with phosphorothioate (PS2) modification within the sense or/and antisense strand of duplex [2,3]. PS2 modification of the internucleotide bond involves the substitution of both non-bridging phosphate oxygen atoms with sulphur and leads to achiral at phosphorus moiety that closely mimic natural RNA and show high nuclease resistance. We applied PS2/PS ‘walking’ methodology for investigation of interactions between the sulphur atoms present at the internucleotide linkages of RNA and human Ago 2 protein. We found, that some positions of modified phosphates at the guide strand (1, 8 and 12 position from the 5' end) are very sensitive and their PS2 modification leads to the loss of siRNA activity. Substitution of PS2 modification by stereoregular phosphates PS RNA (with Rp or Sp configuration at the phosphorus) leads to the recovery of siRNA activity.

References

SW01.S2–98
Prebiotic synthesis of biomolecules in space: key to the quick life origin
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Life originated on the Earth more than 3.5 billion years ago. However, the scientists are still disputing over the possible sources of the life origin. The matter is that life on our planet evolved from the molecular level to the level of bacteria organisms within 0.5–1 billion years, this period being very short for such an important evolutionary step. One of the popular hypotheses asserts that some germs of life have been brought to the Earth from space. A possibility of abiogenesis of synthesis of complex organic compounds (nucleotides and polypeptides) on the surface of comets, asteroids, meteorites and space dust particles in the outer space was tested both in flight and ground-based control experiments. Their results confirm our assumptions that pre-synthesized monomeric units of nucleic acids and proteins could have got to the Earth and thus could have significantly reduced the time period of the evolution process. On the surface of space bodies the scientists have found all kinds of various organic molecules and the components required for their synthesis. We reproduced synthesis of the DNA component adenosine monophosphate under the conditions of the space flight and in specially designed ground-based experiments in order to simulate the space environment. On the Earth the reaction goes in the solution, but there are no solvents whatsoever in space, therefore the experimental pellets were dried in the air and thus formed a pellicle. The major source of energy in the outer space both at present and in the prebiotic period of the Earth history has been the solar ultraviolet radiation of different wavelengths. Therefore, the pellets were irradiated by a powerful ultraviolet lamp in vacuum, and the lunar soil, meteorites Allende and Murchison powder served as a model of the space bodies. The energy of radiation does not promote synthesis alone, it also facilitates decomposition of the compounds, the more powerful the irradiation is, the more extensively the decomposition goes. However, the minerals provided some protection. It seems rather plausible that the synthesis could have taken place on the surface of space bodies at the initial phases of the solar system formation, along with that the chemical evolution (formation and selection of complex molecules) could have started in space. By the time the Earth was formed the chemical evolution might have approached the phase to be followed by the biological evolution. That implies that life on the Earth most probably did not start from the elementary organic molecules synthesis, but commenced from the polymers formation phase. The above assumptions will help the scientists to deeper penetrate into the mystery of the accelerated development of life on the Earth.

SW01.S3 DNA Damage and Repair (I-S3)

SW01.S3–1
Understanding base lesion DNA repair
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The mammalian DNA repair pathway for base lesions, such as 8-oxoguanine, involves excision of the damaged base and then DNA synthesis to fill the excision gap and gap-trimming steps to prepare substrates for downstream enzymes. The topic of base excision DNA repair (BER) will be reviewed during the presentation, along with the hypothesis of ‘substrate-channeling’ of repair intermediates from one step to the next step until repair is complete. The catalytic cycle of DNA polymerase beta is relevant to the substrate-channeling hypothesis because the enzyme plays a central role in BER. New crystal structures of human DNA polymerase beta will be presented and discussed in the context of the catalytic cycle of polymerase beta and the substrate-channeling hypothesis.

SW01.S3–2
Genomic uracil – potent mutagen but normal intermediate in adaptive immunity
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DNA is continuously being damaged by spontaneous decay and exposure to carcinogens. Such damage is cytotoxic and mutagenic. Spontaneous depurination alone accounts for more than 10 000 events per human cell per day, whereas some 100–200 DNA-cytosines are deaminated to mutagenic U:G mismatches per day. Such mismatches are generally processed by essentially error free base excision repair. Importantly, many DNA repair proteins, such as nuclear uracil-DNA glycosylase (UNG2) and mismatch repair proteins are also important for somatic hypermutation (SHM) and class switch recombination (CSR) in B-cells, as part of the adaptive
SW01.S3–3
Nucleotide excision repair: damage recognition, complex assembly and its regulation
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Nucleotide excision repair (NER) is a multistep process involved in removal of structurally diverse DNA lesions. NER factors are sequentially assembled into pre- and post-incision complexes but the regulation of NER in vivo is poorly understood. I will discuss three aspects: (i) regulation of damage recognition, (ii) regulation of transition from dual incision to repair synthesis. The WD-repeat protein DDB2 is the first factor to arrive at lesions and essential for efficient recognition of UV-induced lesions in chromatin. How DDB2 promotes global genome NER in chromatin is poorly understood. We identified PARP1 as a novel DDB2-associated factor and showed that DDB2 facilitates poly(ADP-ribosyl)ation of UV-damaged chromatin through the activity of PARP1 resulting in recruitment of the chromatin remodeler ALC1. Depletion of ALC1 renders cells sensitive to UV and impairs repair of UV-induced DNA lesions. Additionally, DDB2 itself is poly(ADP-ribosyl)ated resulting in increased protein stability and a prolonged chromatin retention time. Our data support a model in which poly(ADP-ribosyl)ation of DDB2 suppresses DDB2 ubiquitylation and outline a molecular mechanism for PARP1-mediated regulation of NER through DDB2 stabilization and recruitment of the chromatin remodeler ALC1.

In the prevailing model, NER operates through coordinated assembly of repair factors into pre- and post-incision complexes; however, its regulation in vivo is poorly understood. Notably, the transition from dual incision to repair synthesis should be rigidly synchronized as it might lead to accumulation of unprocessed repair intermediates. Our data reveal an important function for replication protein A in averting further generation of DNA strand breaks that could lead to mutagenic and recombining events.

SW01.S3–4
DNA damage recognition, signaling and repair
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Base excision repair (BER) is a frontline DNA repair system that is responsible for maintaining genome integrity, thus preventing many human diseases including premature aging and cancer, by repairing DNA base lesions and single strand breaks caused by endogenous and exogenous mutagens. The level of endogenous DNA lesions depends on cellular metabolism and exogenous mutagens, and although this level may vary, it is not clear how the levels of BER enzymes are controlled in response to the changing environment. In other words, what are the mechanisms that detect DNA damage specific to BER and regulate its repair. I will therefore discuss our recent findings on the proteins and mechanisms involved in the coordination of BER.

SW01.S3–5
The new activities in repair of apurinic/apyrimidinic sites
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Apurinic/apyrimidinic sites (AP sites) are one of the most abundant lesions in DNA arising spontaneously or as intermediates in base excision repair (BER). AP sites can appear also under the action of ionizing radiation and can be components of cluster-type lesions in DNA. AP sites within nucleosome produce significant amount of DNA–protein crosslinks and generate double-strand breaks, the most deleterious form of DNA damage. Repair of AP sites is one of the central tasks for protecting DNA structure. Apurinic/apyrimidinic endonuclease 1 (APE1) is the major enzyme initiating cleavage of AP sites and their following repair. We discovered that other DNA repair proteins (PARP1, HMGB1, Ku antigen) can interact with AP sites and regulate their processing. These proteins reveal activities in the cleavage of AP sites. Recently we determined a new catalytic activity of human tyrosyl-DNA phosphodiesterase 1 (Tdp1). The original function of this enzyme consists in hydrolysis of phosphodiester linkages between a DNA 3′-phosphate and a variety of DNA 3′-substituents. However Tdp1 is able to catalyze the cleavage reaction of AP site located inside the DNA strand. It results in generation of DNA breaks with the 3′- and 5′-phosphate termini. The removal of the 3′-phosphate is performed by polynucleotide kinase phosphatase (PNKP). PNKP and DNA polymerase β play key roles in this repair pathway and the activity of both enzymes are stimulated by XRCC1 and PARP1. The data suggest a role of Tdp1 in the new AP endonuclease independent BER pathway in mammals. Tdp1 is more active in the cleavage of AP site located inside single-strand or in bubble-DNA structures. AP site, which located opposite to bulky DNA lesion, is hydrolyzed by Tdp1 faster than single AP site located in dsDNA. This new activity of Tdp1 can contribute to repair of AP sites particularly in DNA structures containing ssDNA region or AP site in the context of cluster-type lesions. The mechanisms of repair of cluster–type DNA lesions containing AP sites will be discussed.

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SW01.S3–6
Regulation of transcription-coupled DNA repair
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Nucleotide Excision Repair (NER) is a versatile DNA-repair pathway, which removes a wide variety of helix distorting DNA damages, including UV-induced lesion. Two different lesion recognition pathways initiate NER; transcription coupled NER (TC-NER) and global genome NER (GG-NER). Post translational modifications play an important role in regulating biological processes, including NER. Particularly differential ubiquitination appeared an impor-
tant regulator in both NER sub-pathways, as several proteins in both sub-pathways are ubiquitinated in response to UV-light.

To reveal UV-dependent changes in the ubiquitin proteome (differential protein modifications by ubiquitination) we have performed quantitative SILAC-based proteomic screen by mass spectrometry (MS). Mono- and poly-ubiquitinated proteins (complexes) were isolated by immuno-precipitation, using antibodies that recognize these substrates.

Several NER proteins, known ubiquitination targets in response to UV-light – like DDB2, XPC, CSB and RNAPolymerase2, were identified and illustrate the validity of our quantitative proteomics approach. The most enriched (ubiquitin-bound) protein in response to UV identified in this screen, with unknown function in NER, is KIAA1530. Genetic and cellular studies showed that KIAA1530 is the causative gene for the, until now genetically uncharacterised, UV-sensitive syndrome group A (UVS-A). UVS-A patients are TC-NER deficient, display photosensitivity and mild freckling but without neurological abnormalities, contrasting to the other TC-NER deficient Cockayne syndrome (CS) patients. Live cell imaging and immunofluorescence immuno-precipitation indicate that this UVSSA protein is a novel TC-NER factor. In the absence of UVSSA the key TC-NER factor CSB protein, is polyubiquitinated and degraded in response to UV. An additional MS screen showed that the de-ubiquitinating (DUB) protein USP7 interacts with UVSSA. We showed that USP7 protects CSB against UV-induced proteolytic degradation. Based on these data we propose a model that the ability of RNase H double-mutant cells to survive replication of rNMPs in DNA, and failure to do so causes replication stress inducing agents, cell cycle and proliferation defects. Levels of ubiquitylated PCNA were also evaluated as signal of post-replication repair activation.

Finally, we are developing yeast as a tool to study how the mutations in AGS2, AGS3, AGS4 found in Aicardi-Goutières syndrome patients affect RNase H2 function. Our findings describe a new function for RNase H and PRR in overcoming the obstacles represented by ribonucleotides misincorporated during DNA replication.

**SW01.S3–8**

**Impact of carcinogen-DNA adducts on DNA methylation**

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Benzo[a]pyrene (B[a]P) is a ubiquitous environmental pollutant. It is metabolized in vivo to highly genotoxic dihydrodiol epoxides (B[a]PDE) which bind to DNA purines thus contributing to the initiation of tumorigenesis. In order to assess the epigenetic contribution of B[a]PDE in tumorigenesis we employed site-specifically modified oligodeoxyribonucleotide duplexes containing stereoisomeric B[a]PDE-derived guanine (B[a]PDE-N²-dG) or adenine (B[a]PDE-N⁶-da) adducts of different conformations as substrates of murine DNA methyltransferase Dnm3a and its catalytic domain (Dnm3a-CD). The length of the B[a]P-DNA was varied and a nucleosome with a single B[a]P-modified 145-mer DNA duplex was constructed in order to develop an approach that would be closer to in vivo conditions. The initial rates of methylation by Dnm3a-CD were significantly reduced by the minor groove trans-B[a]PDE-N⁶-dG adducts, regardless of their position in the substrate, by the intercalated cis-B[a]PDE-N²-dG adducts within the CpG site and by intercalated (+)-trans-B[a]PDE-N²-dG adducts adjacent to the CpG site. The same rules were observed in the case of full-length enzyme and in the presence of the Dnm3a regulatory factor, Dnm3L. The B[a]PDE lesions produced only small effect on Dnm3a-CD binding to DNA. The B[a]PDE-DNA adduct stereochemistry-dependent fluorescence enhancement of the B[a]PDE residues was observed upon interaction with Dnm3a-CD. These results suggest that the movement of the Dnm3a-CD catalytic loop and flipping of the target cytosine are disturbed by the B[a]PDE residues when they are in the DNA minor groove. The impact of intercalated adducts arises from the distortion of the CpG site. The formation of non-productive complexes of Dnm3a-CD with B[a]P-DNA is also possible. Hence, the introduction of B[a]PDE-lesions into DNA, especially the introduction of the most abundant and poorly repairable trans-B[a]PDE-N²-dG adducts, leads to potential alterations of de novo methylation efficiencies thus contributing to the genotoxic effects associated with DNA adduct-induced mutagenic mechanisms.

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Tetracycline induces competence in *Mycoplasma gallisepticum*

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Mycoplasmas are one of the smallest gram-positive bacteria capable of living in a cell free medium. *Mycoplasma gallisepticum* have a very reduced genome, approximately one million bp. The main problem while studying these organisms is their inability to undergo almost any basic genetic modifications, such as plasmid-based targeted gene knockout or gene overexpression. This problem is a result of low natural competence.

Up until now most of the works in that filed are dedicated to transposon mutagenesis, which is a random event and does not result in an efficient tool for further researches.

Transformation is the sum of the two processes: exogenous DNA uptake from the environment (competence) and its incorporation in the genome by the recombination. We annotated two genes, *MGA_0337* and *MGA_0022*, which are homologous to the ones responsible for competence in *B. subtilis* (*comEC*) and *S. pneumoniae* (*comA*) respectively.

Previously there have been shown that damaging conditions such as several antibiotics specifically induce competence in several bacteria. Subsequently we found a condition, under which the expression of the genes responsible for recombination and competence is induced. Although ciprofloxacin, due to its DNA damaging activity, was suggested as a competence trigger, it did not affect the expression level of target genes unlike previously shown data for other bacteria. In return 15-min incubation with 8 mg/ml of tetracycline provoked more than 10-fold inductions of the genes responsible both for recombination and competence at the mRNA level in *M. gallisepticum*.

In the future we are planning to prove the effect of competence induction by tetracycline on phenotypic level by transformation of *M. gallisepticum* in different conditions.
tribution of DNA damage-related proteins in chromatin of the or fragile sites have not been fully elucidated. We studied the dis-
characteristics of nuclear sub-compartments such as the nucleolus studied within the whole genome. However, the region-specific DNA damage response is fundamental for genome integrity
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**SW01.S3–13**

**Epigenetics of DNA repair**

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The maintenance of genome integrity is fundamental for proper cellular functions. Genomes are continuously exposed to genotoxic injuries, including UV irradiation and oxidative stress caused by pollutants. Thus, starting an appropriate DNA repair signaling pathway is more then demanding for genome stability. Genotoxic stress generally leads to induction of strand breaks in DNA and especially double-stranded breaks are dangerous in terms of their faulty repair. The result of inappropriate DNA repair is the emergence of mutations or chromosomal translocations leading to cancer progression. Thus, here, we tried to address the function of epigenetic factors, including histone acetylation, phosphorylation and methylation during DNA damage response. In addition, in living cells we analyzed histone code-related proteins and their functional properties in relationship to optimal DNA repair. In tumour cells, we found acetylation-dependent recruitment of BMII and HP1β proteins into locally induced DNA lesions. Moreover, appearance of Polycomb group-related BMII protein and heterochromatin protein HP1β to DNA lesions was ATP-dependent. Changes in histone acetylation and phosphorylation of H2AX we also studied in embryonic stem cells, characterized by acetylation-dependent recruitment of OCT4 protein to DNA lesions, induced in living cells by local micro-irradiation (Bártová et al., PLOS One; 6: e27281; 2011).

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**SW01.S3–14**

**DNA repair events in chromatin of ribosomal genes and chromosome fragile sites**

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DNA damage response is fundamental for genome integrity maintenance. The DNA repair mechanisms have been extensively studied within the whole genome. However, the region-specific characteristics of nuclear sub-compartments such as the nucleolus or fragile sites have not been fully elucidated. We studied the distribution of DNA damage-related proteins in chromatin of the ribosomal genes and the surrounding genome in the context of epigenetic marks levels.

We cultivated the human osteosarcoma line U2OS and mouse embryonic fibroblasts (MEFs) (wild type and deficient in the histone methyltransferases SUV39 h1 and SUV39 h2). After plasmid transfection and fixation, cells were immunohistochemically stained and used for confocal microscopy with SP5-X system (Leica, Mannheim, Germany). Level of the chromatin associated proteins and histone modifications were analyzed by chromatin immunoprecipitation-PCR. Global protein levels were detected by western blot analysis.

The results show that the heterochromatin protein HP1 and PML recognize spontaneously occurring 53BP1- or γ-H2AX-positive DNA lesions throughout the genome. Moreover, 53BP1 nuclear bodies (NBs), which co-localize with other DNA damage related proteins including γ-H2AX, HP1β and PML NBs, also occur within the nucleoli compartments. Irradiation of the U2OS cells with γ-rays increases the degree of their co-localization throughout the genome and the number of individual PML and 53BP1 bodies, whereas global levels of 53BP1 and PML proteins remained unchanged after γ-irradiation as determined by western blots. However, the 53BP1 protein is less abundant in chromatin of ribosomal genes and fragile sites (FRA3B and FRA16D) in γ-irradiated cells. Selected epigenomic marks differed between non-irradiated and γ-irradiated U2OS cells and MEFs in ribosomal genes and fragile sites. H3K9me3 significantly increases in ribosomal promoters and coding regions after exposure of cells to γ-rays. In fragile sites, γ-irradiation induces an increase in H3K9me3, changes the levels of HP1β, and modifies the levels of H3K9 acetylation.

These results confirmed the region-specificity of DNA repair pathways indicated by the specific DNA damage response that differs between the ribosomal genes and fragile sites.

Work was supported by EU project Marie Curie (PIRES-GA-2010-269156-LCS).

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**SW01.S3–15**

**SRCAP chromatin remodeling complex in double-strand break repair**

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The protein complex SRCAP belongs to SWI/SNF subfamily of ATP-dependent chromatin remodeling complexes and specifically to the INO80-class of remodelers. Previous data indicate that the INO80 and TIP60 members of this class modulate DSB repair by modulating double-strand break end processing and loading of Rad51 recombinase, respectively. To reveal if SRCAP complex may also participate in repair, we investigated the effect of SRCAP depletion in the process. Our data indicate that SRCAP knocked-down cells are hypersensitive to the crosslinker mitomycin C (MMC), suggesting defective homologous recombination (HR) repair. Reporter construct assay confirmed impaired Rad51 foci formation suggesting that the remodeler is defective in a late step of the process.

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**SW01.S3–16**

**Polymerase exchange at replication fork stalled at sites of DNA damage in Saccharomyces cerevisiae**

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Damage to DNA is known to cause lesions which result in replication fork stalling. Replicative DNA polymerases are high fidelity polymerases and cannot bypass these lesions. Specialized polymerases called translesion polymerases are known to bypass these lesions by virtue of their low fidelity and rescue the stalled replication fork. But, the mechanism underlying the switch between replicative polymerase and translesion polymerase has so far remained elusive. In this study, we have identified a protein...
that plays a crucial role in this process. Upon DNA damage, the catalytic subunit of replicative polymerase delta, gets degraded in a mechanism which is dependent on the above mentioned protein, which in turn paves the way for translesion polymerase to take over DNA synthesis and carry out lesion bypass.

**SW01.S3–17**

**Novel small molecules that selectively induce transcriptional activity and modulate marks on chromatin**

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DNA methylation is an important epigenetic regulator of transcription activity, and plays a significant role in development, cell differentiation and tumourogenesis. While mechanisms of de novo and maintenance methylation are well studied, active demethylation remains poorly explained. In order to gain a better understanding of this process, we have carried out a chemogenetic screen aiming to find novel small molecules that can reactivate transcription from methylated plasmid DNA. It has resulted in identification of two different chemical series out of the screened 80 000 compounds that restored the expression of the methylated plasmid to unmethylated levels. The screen provided us with a model to study active DNA demethylation. A kinetic, genome wide, massively parallel sequence analysis of GST-MBD pull down gDNA from HeLa cells treated for 24 h with the small molecule IMBMZ-744363 has identified over 2700 regions (138 lie within promoters) as significantly demethylated, comparing to a mock control. Remarkably, one-third of the identified regions contain one or more CREB_EGR1 modules. RNA-seq and pathway analysis showed the involvement of transcription factors EGR1 and CREB1 in the response HeLa cells make to the compound, as well as the activation of kinase cascades. A detailed analysis of EGR1 promoter revealed the responsive element between the first 50 nucleotides to the transcriptional start site of EGR1. Subsequent work has demonstrated that a CREB responsive element (CRE) on the minus strand is required, but not sufficient, for the response to the compound. Importantly, six CpG dinucleotides are present in the sequence of the minimal response element, including one within the CRE; mutation of any CpG results in loss of inducibility. We furthermore confirmed the role of CREB in the induction of EGR1 expression by transfecting a dominant negative version of CREB into HeLa cells, which blocked the effect of IMBMZ-744363. As CREB is regulated by phosphorylation, we characterized the phosphorylation status of ser133 of CREB, which becomes transiently phosphorylated in response to IMBMZ-744363. Next, we have also shown activation of kinase cascades signaling to chromatin in response to IMBMZ-744363. Inhibition of PI3Kinase activity, as well as of aurora A kinase, prevents compound induced upregulation of the EGR1 promoter. We also demonstrate that Akt, a downstream target of PI3Kinase, is involved in the response cells make to IMBMZ-744363. Moreover, we have carried out a western blot analysis of few selected active and repressive histone marks and showed significant changes in their total amount: active marks (H3K4me3, H3K35me3, H3K14ac and panH3ac) are increased within the first hours after IMBMZ-744363 treatment, followed by the rise of the repressive ones (H3K27me3) later.

Understanding how small molecules can change epigenetic status of a cell will contribute to our understanding of the role of DNA demethylation in regulating gene expression, which can then be applied in the epigenetic treatment of cancer therapy, as in changing the cell fate, either by inducing pluripotency in adult cells or promoting differentiation in stem cell populations.

**SW01.S3–18**

**Nucleolin overexpression leads to increased quantity of DNA double strand breaks after etoposide treatment on HeLa cells**

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The use of DNA topoisomerase II inhibitors (topoisomerase poisons) in anticancer chemotherapy does not infrequently leads to development of the so-called ‘treatment-related’ or ‘secondary’ leukemias associated with chromosomal translocation (8;21) (q22;q22) affecting AML1 and ETO genes. Under the action of topoisomerase poisons DNA double strand breaks are formed in the nuclei which can lead to further chromosomal rearrangement. Obviously this requires direct ‘contact’ of DNA break’s flanks of different chromosomes in the same nuclear compartment where damaged DNA repair should occur. Previously we have shown that treatment of Jurkat cells with DNA topoisomerase II inhibitor etoposide leads to increase of the probability of localization of ETO gene on the surface of the nuclei as well as enrichment of major nucleolar protein nucleolin within ETO containing chromatin locus. We hypothesized that nucleolar proteins may be involved in the DNA double strand breaks repair and that surface of the nuclei is the compartment where chromosomal translocations might occur.

The aim of this work was to determine the possible role of nucleolin in the processes following treatment of cells with etoposide. By means of transient transfection we have obtained HeLa cells with overexpression of nucleolin. DNA double strand breaks were detected by using of neutral ‘comet-assay’ and pulsed-field gel electrophoresis.

Analysis of the average molecular weight of DNA fragments formed after cell treatment with etoposide shown that the fragmentation of DNA in cells with nucleolin overexpression used to be higher than in control. Therefore overexpression of nucleolin leads to increased sensitivity of HeLa cells to etoposide treatment. And since we know that nucleolin have the activity of histone chaperone and might be involved in chromatin remodeling we can assume that its overexpression can lead to a general or local chromatin decondensation and consequently increases the number of sites on the DNA available for binding and cleavage by DNA topoisomerase II. Further information about the possible functions of nucleolar proteins in the formation and repair of DNA double strand breaks can be useful for understanding the mechanisms of chromosomal rearrangements, as well as to find new treatments for oncological diseases.

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**SW01.S3–19**

**The impact of dUTPase expression on genome integrity**

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Accurate and stable DNA synthesis requires the precise regulation of dNTP pool. This regulation involves (i) meeting the requirements of cell cycle or DNA repair dependent DNA syn-
thesis, (ii) the maintenance of the proper ratio of dNTP components, and (iii) the elimination of the modified nucleotides from the pool. Perturbed regulation results in the appearance of DNA lesions compromising genome integrity mostly by base excision (BER) or mismatch repair. Uracil bases are frequent lesions that can occur in DNA. dUTPase has the essential role to prevent dUMP incorporation into DNA, and an uracil DNA glycosylase, UNG, a component of BER is responsible for uracil removal from the genome.

From previous observations we learned that the fruit fly lacks UNG from the three components and possess only a stoichiometric expression of dUTPase. We showed that the uracil content of DNA depends only on dUTPase expression in this organism. I also examined the transcriptional regulatory mechanism that forms dUTPase expression pattern. My results suggest that DRE motifs located in dUTPase promoter may be responsible for a cell cycle dependent expression, but there might be a DRE independent gonad-specific regulation as well. We also showed that compromising dUTPase expression in proliferating tissues causes cell autonomous tissue degradation during pupal stages. Furthermore, we showed genomic uracil accumulation, DNA fragmentation, and DNA damage response activation these tissues.

SW01.S3–20
Application of repair enzymes to improve the quality of the DNA template in PCR amplification of degraded DNA
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Despite the fact that DNA in living organisms is used as the main carrier of genetic information, the chemical stability of this molecule is limited. In living organisms, such DNA damage can lead eventually to the emergence of mutations, tumors, and contribute to aging. During the life of the organism, the repair systems resist the accumulation of damage in DNA, but with the death of the organism, these processes cease working, and the accumulation of DNA damage becomes irreversible. The accumulation of damage in DNA can be a problem when it is necessary to analyze its sequence. For example, the efficiency of PCR is sharply reduced if the template is subjected to oxidation or apurinization. This is particularly actual in studies of ‘ancient DNA’ and DNA in the forensic practice. We are developing a system in which repair enzymes are used to improve the quality of degraded DNA matrices before PCR.

SW01.S3–21
Characterization of the in vivo functions of PrimPol, a novel TLS primase-polymerase
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Genomic integrity depends critically on the fidelity and efficiency of DNA replication. The catalytic site of the replicative DNA polymerases is compact and intolerant of DNA lesions such as thymine dimers induced by UV light. As a consequence, translesion DNA synthesis (TLS) is is used to bypass such lesions and facilitate replication fork progression. We have identified PrimPol, a yet uncharacterized protein encoding a primase domain of the AEP (Archaean-Eukaryotic Primase) family. In vitro, PrimPol is able to bypass oxidative 8-oxo-G and UV lesions in DNA, suggesting a role in TLS. PrimPol localizes to both the nucleus and mitochondria indicating it may play important roles in genome maintenance in both compartments. In order to investigate the role of PrimPol in vivo, we have generated mice lacking its expression. PrimPol−/− mice exhibit increased genomic instability that is exacerbated upon UV damage or aphidicolin treatment. Consistent with this, damaged cells show sustained phosphorylation of Chk1 and p53 in the absence of PrimPol. Additionally, the expression of PrimPol is highest in UV sensitive tissues including the eye and skin. To validate the in vivo function of PrimPol we analyzed histological changes in the skin of mice upon acute UV exposure. UV damaged skin in PrimPol−/− mice showed marked epidermal hyperplasia compared to wild type. Xeroderma pigmenotum (XP) is a disease caused by the mutation of the TLS polymerase Pol η, responsible for the repair of TT dimers. Depletion of Pol η in PrimPol−/− MEFs leads to synergistic sensitivity to UV damage. We have also observed increased mitochondrial DNA copy number in PrimPol−/− mice in tissues with high metabolic turnover implying that loss of PrimPol may impair the repair of oxidative damage leading to mitochondrial stress. Based on these findings, we hypothesize that PrimPol functions in TLS by bypassing TT dimers after UV irradiation and functions in parallel to Pol η. This activity is likely required for the maintenance of both the mitochondrial and nuclear genomes in response to UV and oxidative base damage. PrimPol knockout mice provide a model system for understanding the potential role of this enzyme in the etiology of sunlight induced skin cancers and premature ageing induced by oxidative stress. Current results from the ongoing characterization of these animals will be presented.

SW01.S3–22
dUTPase based switch controls transfer of virulence genes in order to preserve integrity of the transferred mobile genetic elements
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dUTPases ubiquitously regulate cellular dUTP levels to preserve genome integrity. Recently, several other cellular processes were reported to be controlled by dUTPases including the horizontal transfer of Staphylococcus aureus pathogenicity islands (SaPI). SaPIs are mobil genetic elements that encode virulence enhancing factors e.g. toxins. Here, phage dUTPases were proposed to counteract the repressor protein (Stl) and promote SaPI excision and transfer. A G protein-like mechanism was proposed which is unexpected in light of the kinetic mechanism of dUTPase.

Here we investigate the molecular mechanism of SaPI transfer regulation, using numerous dUTPase variants and a wide range of in vitro methods (steady-state and transient kinetics, VIS and fluorescence spectroscopy, EMSA, quartz crystal microbalance, X-ray crystallography).

Our results unambiguously show that Stl inhibits the enzymatic activity of dUTPase in the nM concentration range and dUTP strongly inhibits the dUTPase: Stl complexation. These results identify Stl as a highly potent dUTPase inhibitor protein and disprove the G protein-like mechanism. Importantly, our results clearly show that the dUTPase:Stl complex is inaccessible to the Stl repressor. Unlike in small GTPases, hydrolysis of the substrate nucleoside triphosphate (dUTP in this case) is required prior to the interaction with the partner (Stl repressor in this case). We propose that dUTPase can efficiently interact with Stl and induce SaPI excision only if the cellular dUTP level is
low (i.e. when dUTPase resides mainly in the apo enzyme form) while high dUTP levels would inhibit SaPI transfer. This mechanism may serve the preservation of the integrity of the transferred SaPI genes and links the well-known metabolic role of dUTPs to their newly revealed regulatory function in spread of virulence factors.

**SW01.S3–23**

Zinc finger nucleases generate DNA double strand brakes and modification in *Chlamydomonas reinhardtii*

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The green microalga *Chlamydomonas reinhardtii* is a valuable model organism for the study of fundamental biological processes such as photosynthesis, metabolism, phototaxis, flagella assembly, circadian rhythmicity, the cell cycle and mating. Important, *Chlamydomonas* preserves characteristics of plant and animal cells and is regarded as their common ancestor. Similarity with mammalian cells makes this alga a volatile model for human ciliopathies that is regarded as their common ancestor. Similarity with mammalian cells making this alga a valuable model for human ciliopathies that result from defects in primary cilia. The computer analysis of *Chlamydomonas* genome databases has revealed the central homologous recombination (HR) and DNA non-homologous end joining (NHEJ) function in *Chlamydomonas* are still poorly understood. Similar as in higher eukaryotes, when exogenous DNA is introduced into *Chlamydomonas* cells, HR is three orders of magnitude less efficient than NHEJ which makes site-specific mutagenesis through gene targeting difficult. For site-specific genomic modification and characterization of HR and NHEJ products we designed, constructed and used zinc finger nucleases (ZFNs). We demonstrated by targeted disruption of the *CHRI* gene (encoding channelrhodopsin-1) that in *Chlamydomonas* modular assembly-based ZFNs can introduce site-specific double stranded breaks (DSB) and stimulate both NHEJ and HR with the supplied template DNA. We found that about 1% of the clones expressing active *CHRI*-specific ZFN contained a mutated *CHRI* locus. If modified template DNA was supplied, *CHRI* alterations were exactly copied through a pathway best described by the synthesis-dependent strand annealing mechanism. Without template DNA DSB repair went through mutagenic NHEJ. Mutations generated through this pathway included long insertions copied from the chromosomes or deletions between short repeats. Other experiments on analysis of mutations generated by earlier described ZFNs Zif268 or GZ1/GZ3 confirmed that the most frequent mutations were deletions from 8 until longer than 400 bp which took place between repeated sequences from 2 (GC) until 8 bp (GGTGGGCCC). Our data suggest that in *Chlamydomonas* an alternative microhomology-mediated end-joining pathway is high efficient: this mechanism is initiated by ssDNA resection, mediated by base pairing between microhomologous sequences and Ku-independent. For further study of DSB repair and the gene targeting machinery in *Chlamydomonas*, ZFNs specific to proteins of HR and NHEJ pathways will be designed and validated for site-specific mutagenesis.

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**SW01.S3–24**

Comparative analysis of interaction of PARP1 and PARP2 with apurinic/apyrimidinic DNA

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One of the most frequently formed DNA damages (about 10 000 lesions per mammalian cell per day) is apurinic/apyrimidinic (AP) site. Unrepaired, AP sites present mutagenic and cytotoxic consequences to the cell. UV-light or X-ray irradiation dramatically increase the number of AP sites. AP sites in mammalian cells are generally processed via functioning of base excision repair (BER) system. One of key regulatory proteins in BER is PARP1. Its role in response to DNA damage has been widely illustrated, the contribution of another DNA-dependent PARP, PARP2, has not been studied in detail. It is the closest homolog of PARP1.

In this work we studied interaction of PARP2, with AP sites and several BER proteins taking part in the AP sites processing. We demonstrated the capability of PARP2 to interact with abasic sites forming stable complexes PARP2-abasic DNA. The repair of AP sites in canonical BER is initiated through hydrolysis by AP endonuclease 1 (APE1) or β-elimination via the activity of DNA glycosylases and other enzymes with associated AP lyase activity. PARP1 possesses 5′-dRP/AP lyase activity. In contrast, PARP2 is unable to cleave abasic sites, but like PARP1, it displays 5′-dRP activity. PARP1 and PARP2, although to a lesser extent, are able to inhibit the APE1 activity, with inhibition being regulated by poly(ADP-ribose)ylation. Like PARP1, PARP2 interferes with the activity of downstream BER enzymes – DNA polymerase β and flap endonuclease 1.

Taken together, our obtained results testify to probable additional regulation of BER by PARP2. Study of the mechanisms of PARP involvement in BER can be essential for developing new drugs for selective inhibition of these proteins.

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**SW01.S3–25**

RecA730 dependent suppression of DNA repair deficiency in RecA loading mutants of *Escherichia coli*

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Homologous recombination is essential for DNA repair and the maintenance of genome integrity. The most dangerous DNA damage are double-strand breaks (DSBs) which can arise during normal cell cycle or as a consequence of the action of DNA damaging agents [1]. In *Escherichia coli*, repair of DSBs occurs by homologous recombination. The central part of recombination process is binding of RecA protein to ssDNA, i.e. production of RecA filament. DSBs can be processed into a RecA filament by the action of three enzymatic activities: helicase, 5′-3′exonuclease and RecA loading onto ssDNA. These activities are provided by the RecBCD enzyme in wild type cells or by the RecF pathway gene products in the *recBC sbcBC( D)* cells [2].

The RecA730 mutant protein binds to ssDNA more efficiently than SSB protein, and consequently it is able to produce a RecA filament without the help of RecFOR mediators [3]. We wanted to test whether the recA730 mutation can suppress DNA repair defi-
ciency in derivatives of recB1080 mutant, i.e., in recB1080 recF (OR) and recB1080 recJ. The RecB1080CD enzyme has abolished nuclease and RecA loading activities, but retains helicase activity [1][4]. In recB1080 mutant, the DSBs are repaired by the hybrid recombination pathway where helicase activity is provided by RecB1080CD enzyme, 5'-3' exonuclease by RecJ protein and RecA loading by RecFOR complex [5]. We looked on cell survival after γ-irradiation, and found that recA4730 mutation suppresses DNA repair deficiency in recB1080 recF(OR) background where the defect is at the level of RecA loading, but not in recB1080 recJ background where the defect is at the level of nuclease activity.

References

SW01.S3–26
Single-molecule studies of dsDNA properties using optical tweezers
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Optical tweezers are scientific instruments that allow to control and manipulate dielectric objects of micron and submicron size (e.g. plastic and glass microspheres, bacteria, eukaryotic cells) in aqueous solution and which are capable of measuring forces applied to these objects in the range of 0.1–100 pN. Optical trapping was firstly reported by Arthur Ashkin in 1970 [1].

Optical trap is generated by a laser beam brought into sharp focus by a microscope objective of high numerical aperture. In our lab at the SPbSTU Institute for Nanobiotechnologies, we have constructed a double-trap optical tweezers apparatus equipped with both steerable and fixed traps. Steering is performed using a piezo mirror and a system of multiple lenses.

We have combined optical trapping with microfluidics to develop a method for studying the elastic properties of DNA at the single-molecule level. A 1 phage DNA molecule with biotinylated ends is attached to two streptavidin-coated polystyrene microspheres to form a ‘dumbbell’. The DNA is stretched by manipulating the attached microspheres with optical tweezers and the response to stretching is registered. A specially designed 4-channel flow chamber allows for rapid changes in reaction conditions by moving the dumbbell between different channels, allowing for studies of DNA-protein interactions in real time.

We have successfully tested this method by studying the changes of DNA properties upon binding of well-known DNA-binding agents such as YOYO-1 intercalating dye and RecA protein. Present work is focused on studying interactions of TIP49 human proteins and dsDNA.

Reference

SW01.S3–27
Structural and biochemical characterization of the Rod-Zwilch-ZW10 (RZZ) complex
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Background: The 3-subunit Rod-Zwilch-Zw10 (RZZ) complex is a crucial component of the spindle assembly checkpoint (SAC) in higher eukaryotes. It is required for kinetochore localization of the Mad1-Mad2 checkpoint complex and of the microtubule motor dynein, thus contributing to kinetochore-microtubule attachment as well as to the dynein-dependent stripping of SAC components upon checkpoint satisfaction. How the RZZ fulfills these different roles and integrates signals from the kinetochore-microtubule interface remains unclear.

Results: We study the organization and function of the RZZ complex by using a multidisciplinary approach that combines structural biology and biochemistry. We have reconstituted recombinant versions of the RZZ and of its subunits. Negative-stain single-particle electron microscopy revealed that the RZZ complex has a two-fold symmetry and an elongated but rigid shape. Biochemical analysis shows that ZW10 forms a stable complex with the KMN components Knl1 and Zwint, which is probably important for kinetochore recruitment of the RZZ.

Conclusions: Our studies suggest a mechanism by which the RZZ complex may act as a functional link between the SAC and the microtubule attachment machinery and pave the way to a detailed structural and functional characterization of the RZZ complex.
generated under thymine starvation. Furthermore, we found that TLD is suppressed in oriC deletion strains. We show that in these genetic backgrounds the efficiency of initiation of chromosomal replication was drastically reduced and the Chromosomal Initiation Capacity (ChiC) was almost abolished. The results presented here indicate that, under thymine starvation, initiations of chromosome replication do indeed occur and they correlate with TLD. These observations reveal the initiation events as critical targets to develop new therapies improving the lethal effect of the treatment mimicking thymine starvation.

**SW01.S3–29**
The involvement of Cockayne syndrome B protein in base excision repair

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Cockayne syndrome (CS) is a rare human disorder characterized by pathologies of premature aging, neurological abnormalities, sensorineural hearing loss and cachectic dwarfism. CS is caused by mutations in CSB (80% of the cases) and CSA (20%) genes which participate in DNA repair and transcription. We have characterized the interaction of CSB with other proteins to determine the molecular pathways in which it functions. We have identified novel physical and/or functional interactions of CSB with several proteins involved in nuclear and mitochondrial base excision repair (BER) pathway for restoring damaged DNA bases, including 8-oxoguanine DNA glycosylase 1 (OGG1), apurinic/apyrimidinic endonuclease 1 (APE1) and endonuclease VIII-like 1 and 2 (NEIL1 and NEIL2). CSB plays a role in the repair of formamidopyrimidines (2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine (FapyA)) in the nuclear DNA by interacting with and stimulating NEIL1 or NEIL2 incision activities. Moreover, the oxidized DNA bases, such as 8-hydroxy-7,8-dihydroguanine (8-oxoG), 7,8-dihydro-8-oxoadenine (8-oxoA), FapyG or FapyA accumulates in the DNA of CSB-deficient cells, and in brain and kidney of CSB-knockout mice. Mitochondria have an independent BER pathway to protect the integrity of mitochondrial DNA (mtDNA). CSB is present in mitochondria where it associates with mtDNA in nucleoids and translocates to mitochondria after oxidative stress. That suggests, CSB may also be involved in the recruitment and stabilization of BER proteins to the repair complexes associated with the inner mitochondrial membrane. CSB-deficient cells have decreased levels of mitochondrial 8-oxoG, uracil and 5-OHU incision activities, and decreased AP endonuclease activity. In summary, CSB interacts with and affects the function of numerous proteins involved in nuclear and mitochondrial BER, and it seems conceivable that some (if not all) of these interactions may be important for efficient genome maintenance. Moreover, these findings provide a novel basis for understanding the complex phenotype of this debilitating disorder.

**SW01.S3–30**
Caffeine enhances proapoptotic activity of sodium butyrate, gamma-IR, UV-C and cisplatin in HeLa cells

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Caffeine is the most wildly consumed psychoactive substance producing complex pharmacological actions. Exposure of cells to DNA damaging agents such as UV-C, gamma-IR, and chemotherapeutic agents – cisplatin, induces an arrest in their cell cycle progression to allow time for DNA repair. Caffeine is a PI-3 kinase inhibitor and affects tumor cells through various pathways – PTEN, AKT, Bcl associated X-protein (BAX), caspase 3. We have investigated the molecular and cellular mechanisms underlying the proapoptotic effect of caffeine on HeLa cells treated with gamma-IR, UV or cisplatin. We show that pretreatment with caffeine enhances the potency of the DNA-damaging agents through abrogation of the G1/S or G2/M checkpoints and inducing apoptosis. The epigenetic silencing of tumor suppressor genes induced by overexpression of histone deacetylases (HDACs) plays an important role in carcinogenesis. Thus HDAC inhibitors (like sodium butyrate) have emerged as supplementary therapeutic agents, since they can block the activity of HDACs, restore the expression of some tumor suppressor genes and induce cell differentiation and apoptosis. Interestingly, we found that HeLa cells treated with caffeine and sodium butyrate showed G1/S block and a three-fold increase of the mortality rate in comparison with cells treated with caffeine only. Western blot analysis revealed this effect was accompanied by a decrease of histone H4 acetylation levels and activation of caspase 9 and caspase 3. These findings are of interest for the development of new therapeutic strategies using HDAC- and PI-3 kinase inhibitors in combination with common DNA-damaging agents. It might permit use of lower therapeutic doses and reduction of adverse side effects when treating cervical carcinomas.

**SW01.S3–31**
CRP, IL6, IL10 levels and CRP polymorphism in patients with pancreas cancer

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**Background:** Aim of this study was to determine relation with pancreas cancer and inflammation. CRP was one of the important markers at inflammation which studied polymorphism of CRP at pancreas cancer.

**Patients and Method:** We conducted study within the Oncology hospital of Gaziantep, measuring blood levels of C-reactive protein (CRP), interleukin-6 (IL-6) and interleukin 10 (IL-10) in 50 pancreas cancer cases and 50 matched controls. CRP blood levels were assessed by nephelometric and IL-6, IL-10 levels by chemiluminescence assays. DNA was extracted from blood samples, and the C-reactive protein (CRP) 1846 C>T and 5922 A>T genetic polymorphisms were investigated using by polymerase chain reaction-restriction fragment length polymorphism.

**Results:** Serum CRP and IL-10 levels were significantly higher than control group. IL-6 levels were different than those in the control group (p = 0.058). It was found that the 1846 CT polymorphism was associated within patients and control groups. (p = 0.030). The 5922 T/A polymorphism showed a significantly higher frequency of pancreas cancer matched to control (p = 0.009).
Conclusion: These data suggest that both of this region polymorphism was associated with pathogenesis of pancreas cancer.
and a tyrosine residue as well as a variety of other DNA 3’ damaged termini. Recently we determined that human Tdp1 interacts with AP sites and catalyzes the AP-site-cleavage reaction to generate breaks with the 3'- and 5'-phosphate termini. The removal of the 5'-phosphate is performed by polynucleotide kinase phosphatase (PNPK). PNPK and PolⅢ play key roles in this repair pathway and the activity of both enzymes are stimulated by XRCC1. The data suggest a role of Tdp1 in the new AP-endonuclease independent BER pathway in mammals. Tdp1 is more efficient in the cleavage of AP site in single-strand or bubble DNA structures. AP site that is opposite to bulky DNA lesion is hydrolyzed by Tdp1 faster than single AP site located in dsDNA. Tdp1 is also able to cleave synthetic analogs of AP site – tetraphosphuran and diethylthiophosphoryl (decandiol and diethylenglycol) in contrast to enzymes possessing AP-lyase activity (e.g. NellI and EndoIII). Tdp1 mutants (SCAN1 and H263A) can bind the AP-site-containing DNA but do not reveal endonuclease activity. This new activity of Tdp1 can contribute to repair of AP sites particularly in DNA structures containing ssDNA region or AP site in the context of cluster-type lesions.

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**SW01.S3–36**

**Kinetic features of AP-site cleavage by Apn1 from Saccharomyces cerevisiae and its H83A mutant in base excision repair**

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Under exposure of endogenous and exogenous factors DNA damages continually arise. One of frequent occurrence damages is apurinic/apyrimidinic (AP) site. AP-sites are very mutagenic and genotoxic. They are repaired mainly by AP-endonucleases in a base excision repair pathway. AP-endonuclease 1 from *Saccharomyces cerevisiae* (Apn1) is known to possess the major apurinic/apyrimidinic activity incising phosphodiester backbone 5’ to the AP-site. Up to date the spatial structures of Apn1 and its complex with DNA are not reported. Therefore, the kinetic features of the interactions of Apn1 and its mutant form with DNA and conformational dynamics of the reactants would shed light on the role of key aminoacid residues involved in recognition and catalysis.

The conformational dynamics of DNA substrates during AP-endonuclease catalytic cycles were investigated by measuring 2-aminopurine (2-aPu) and pyrrolocytosine (PyrC) fluorescence. The fluorophores were incorporated in a damaged strand or in a complementary strand of DNA duplex to detect conformational changes of each strand. Pre-steady-state kinetics of the repair process was studied by stopped-flow method and rate constants of each elementary step were calculated by global non-linear regression fitting.

The experimental data analysis has revealed significant differences in catalysis by *S. cerevisiae* Apn1 and Apn1 H83A. The location of 2-aPu relative to the damage was shown to have no influence on substrate cleavage by Apn1; but in the case of Apn1 H83A location of 2-aPu is essential. Conformational changes of DNA substrate were shown to take place in the course of specific complex formation. When interacting with the enzymes, both DNA strands are involved in the recognition process. The initial conformation of double-stranded nucleic acid is of great importance for the formation of the proper enzyme-substrate complex.

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**SW01.S3–37**

**Influence of thymidine glycol on DNA mismatch repair**

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The mismatch repair (MMR) system plays a crucial role in the prevention of replication errors and in the correction of some oxidative damages of DNA bases. In the present work the potential repair of the most abundant oxidised pyrimidine lesion, 5,6-dihydro-5,6-dihydroxythymidine (thymidine glycol, Tg) by *E. coli* MMR system has been investigated. In a partially reconstituted MMR system using MutS/MutL/MutH proteins we observed that G/Tg-containing plasmid DNA was impaired to provoke incision and thereby initiation of MMR. Compared to this DNA with A/Tg pair could be a better but still poor substrate for MMR system. To reveal which steps in the initial phase of the MMR were affected, the interactions of MutS with A/Tg- and G/Tg-containing DNAs were investigated in details. MutS had a 3–6 times lower affinity to Tg-containing DNAs compared to G/T-duplex (the *Kd* value was approximately 7.2 nM). The *Kd* value for duplex containing G/Tg pair (approximately 45 nM) and A/Tg-duplex (approximately 25 nM) were similar as for the canonical DNA (approximately 33 nM). The higher affinity of MutS to A/Tg-duplex is in agreement with the slightly higher DNA incision by MutH/MutL/MutS complex as compared with native plasmid. Using a recently developed FRET assay to monitor DNA kinking and DNA binding orientation by MutS, we show that despite of reduced affinity to oxidised DNA lesion the MutS orientation on the DNAs with G/Tg or A/Tg pair and with G/T mismatch was the same. However, in the case of both Tg-containing DNAs the characteristic DNA kink was not observed. The lack of such a kinking can be explained by the disruption of specific DNA binding with MutS. Furthermore DNAs containing G/Tg and A/Tg pairs did not stimulate the nucleotide exchange in ATPase domain of MutS as effective as that observed for DNA with a G/T pair. In summary, the MutS transformation to an active conformation (sliding clamp) and hence the interaction with MutL is likely impaired. Taken together, our results demonstrate that thymidine glycol residue in DNA probably not repaired/processed (or repaired only with a small extent) by the MMR system. The lack of repair can be attributed to the failure of MutS to interact with modified DNA containing nonplanar thymine glycol.

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The role of yeast *Saccharomyces cerevisiae* HSM3 and HSM6 genes in DNA repair, mutagenesis and chromatin modifications

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The investigations of the last decade revealed the substantial role of chromatin as the main factor of DNA stability and genome integrity. Chromatin is now discussed to be the interlink between fundamental processes occurring in the living cell, such as DNA replication, transcription, repair and mutagenesis. Moreover, it was shown that abnormality of chromatin structure causes the appearance and development of different types of cancer and genetic diseases in human organism. The same time, precise chemical post-translation chromatin modifications are strictly required for cell cycle control, signaling and internal cell transport.

In our lab the collection of specific mutants was obtained. The *hsm3* and *hsm6* mutants are remarkable for high spontaneous mutation rate but are not sensitive to different DNA-damaging agents’ lethal actions. The product of the *HSM3* gene was, as first, referred to the proteins of DNA mismatch repair pathway; the product of the *HSM6* gene was, in the beginning, related to polymerase family. We examined the properties and functions of the *HSM3* and *HSM6* genes and the proteins they encode.

For the *HSM6* gene we established that that is the allele of *PSY4* gene, which product is Psy4p – the subunit of Pph3-Psy2-Psy4 complex taking part in histone gamma-H2A dephosphorylation. Psy4p interacts with Mec1p, Rad53p and Rad9p – main checkpoint proteins activating Dun1-kinase checkpoint pathway which, by turn, regulates cell dNTP pool by effecting on ribonucleotide reductase (RNR) activity. Studying the interactions between *hsm3* mutations and mutations in primary genes of homologous recombination and postreplicative DNA repair pathways we established the key role of Hsm3 protein in these DNA repair mechanisms. We examined the interactions between *hsm3* mutations and the mutations in *MPH1* and *SHU1* genes shown to affect on D-loop formation. The damaged Hsm3p causes D-loop destabilization and prevents its processing.

We determined that the Hsm3p has as minimum three domains and C-terminal part of Hsm3p is responsible for the induced and spontaneous mutagenesis control. It was known that the Hsm3p has chaperone functions and binds with the subunits Rpt1, Rpt2 and Rpn1 of the proteasome complex. We showed that mutations in C-terminal part of Hsm3p did not affect on the proteasome assembling.

We genetically proved that the Hsm3p interacts with the subunits Hat1p, Hat2p and Ht1p of HAT-B/NuB4 histone H4 acetyltransferase complex. The same time Hsm3p is able to affect indirectly on the dNTP pool.

# SW01.S3–39

Dissecting base excision: new insights into the mechanism of lesion recognition by formamidopyrimidine-DNA glycosylase

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Oxidation of biomolecules presents a perpetual problem for all aerobic life. In bacteria, formamidopyrimidine-DNA glycosylase (Fpg) removes oxidatively damaged purines from DNA, keeping it free of premutagenic lesions such as 8-oxoguanine (oxoG) and formamidopyrimidines. The X-ray structure of Fpg from several species is known, yet the substrate recognition and catalysis by this enzyme is a dynamic multistep process, many steps of which remain poorly characterized. As follows from the structure, Fpg binds and bends DNA, flips the damaged base into the enzyme’s active site, inserts several amino acid residues into the resulting void in DNA, and catalyzes hydrolysis of the N-glycosidic bond and elimination of the phosphates flanking the lesion. We have combined computational analysis, site-directed mutagenesis and substrate perturbation to dissect the mechanism of several steps in the Fpg-catalyzed reaction. Individual domains of *E. coli* Fpg have been purified; of these, the C-terminal helix–two turn–helix–zine finger domain retained the ability to bind DNA while the catalytic N-terminal domain did not. The analysis of co-conservation of the residues in the Fpg family, followed by site-directed mutagenesis, protein melting, and enzyme kinetics, allowed us to identify several salt bridges important for the stability of critical structural motifs in Fpg. The stacking energy of oxoG within different sequences was determined, and stopped-flow kinetics with fluorescence detection was used to correlate this energy with the efficiency of individual steps along the reaction pathway. Molecular dynamics was used to explain the preference of Fpg to excise oxoG from pairs with C but not from pairs with A. Steered molecular dynamics was performed to suggest the residues promoting the conformation changes during the substrate recognition by Fpg, and their roles were confirmed using site-directed mutagenesis and enzyme kinetics. Finally, kinetics of cleavage of DNA substrates containing single-strand breaks in defined positions revealed several phosphodiester bonds contributing to the stabilization of the reaction transition state.

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# SW01.S3–40

DNA damage response in normal human T cells

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DNA damage response (DDR) is a signalling pathway operating at checkpoints within the cell cycle. It protects cells against DNA damage by leading to: (i) temporary cell cycle arrest and DNA repair, (ii) cell death if lesions are too severe, (iii) permanent cell cycle arrest and cellular senescence when DNA damage is within telomeres. T cells circulating in the blood can be quiescent or stimulated by a pathogen. Stimulation of T cells (CD3+CD28+) leads to the activation-induced cell death, that is, proliferation followed by massive cell death and survival of memory cells. T cell stimulation leads also to senescence manifested as accumulation of non-dividing CD8+CD28− subpopulation of CD3+ cells. In this study we asked two questions: whether quiescent (non-cycling) T cells can activate DDR and whether T cell stimulation is followed by DNA damage and DDR that leads to T cell senescence. To answer these questions we used T cells isolated from healthy young donors. We treated quiescent cells with a DNA damaging agent, etoposide, or activated them with a mitogen, what resulted in the exhaustion of cell proliferation and accumulation of CD8+CD28− cells. We showed that etoposide, which is a topoisomerase II inhibitor, could influence transcription and was able to activate DDR in resting human T cells by inducing phosphor-
ylation of ATM and its substrates, H2AX and p53. This subsequently led to the activation of PUMA, caspases and to apoptotic cell death. Next, we used an ATM inhibitor, KU 55933, which has been shown previously to be a radio/chemo-sensitizing agent. Pretreatment of resting T cells with KU 55933 blocked phosphorylation of ATM, H2AX and p53, which, in turn, prevented PUMA expression, caspase activation and apoptosis. However, etoposide-induced DNA damage in resting T cells was not influenced by KU 55933 as revealed by the FADU assay. Altogether our results show that KU 55933 blocks DDR and apoptosis induced by etoposide in normal resting T cells. We have also shown that curcumin, a natural agent with anticancer potential, can induce apoptosis of normal resting human T cells that is not connected with DNA damage. Our results also revealed that T cell stimulation can induce DNA damage and DDR which can be the primary reason of T cell senescence in vitro.

SW01.S3–41
Das13 mutation in bacteriophage T4 RNase H increases its exonuclease activity
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Bacteriophage T4 RNase H, a member of FEN-1 endonuclease family, possesses both flap endo- and 5′–3′ exonuclease activities on double-stranded DNA. The mutations mapped to rnh gene encoding for RNase H (known also as das mutations) were selected as specific suppressors of DNA arrest phenotype caused by defect in 46 and/or 47 genes. Recently, it was shown that product of these genes is a nuclease complex, which is an analog of eukaryotic Mre11/Rad50. We have sequenced the phage T4das13 rnh gene and found two mutations, which lead to amino acid substitutions V43I and L242I. The analysis of known 3D structures of phage T4 RNase H has shown that both mutations do not participate in formation of the active center or DNA-binding sites. In order to determine whether these mutations affect the activity of the enzyme on dsDNA the nuclease assays were carried out. We tested both wild-type protein and three mutants (Das13 with both substitutions, and proteins with single substitutions V43I or L242I) for the nuclease activity in vitro. It was found that the activity of single mutant V43I and Das13 protein was almost the same and significantly increased, while the activity of L242I mutant was similar to the wild type protein. To study the impact of V43I (and Das13) substitution on endonuclease and exonuclease activity the nuclease assay on synthetic substrates was performed. It was found that exonuclease activity of V43I mutant on GAP-substrate was in 10 times higher compared to the wild type RNase H, whereas its endonuclease activity on Y-substrate remained unchanged. In addition, in our experiments, the mutant phages, carrying V43I substitution in RNase H were able to suppress conditionally lethal mutation in gene 47.

Taken together, our findings suggest that the single mutation V43I is sufficient to increase exonuclease activity of phage T4 RNase H and to suppress DNA arrest phenotype caused by defect in gene 47.

SW01.S3–42
An inter-species landscape of DNA repair proteins based on extreme metagenomes and repeated HMM profiling
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Next generation sequencing technologies made possible the discovery of numerous new microbe species in environmental samples. Perhaps it is more important to discover new genes of remarkable functions and properties. In this study, we identified proteins in DNA repair in well-known organisms (i.e. proteins in base excision repair, nucleotide excision repair, mismatch repair and DNA break repair); next we applied multiple alignments (with CLUSTAL) and then built HMMER profiles for each protein separately, across the well-researched (model- and non-model organisms); next, using public depositories of metagenomes, originating from extremely hot, extremely cold, or extreme pH environments, we identified DNA repair genes in the samples. Note, that the phylogenetic classification of the samples are not typically available. We hypothesized, that some very special DNA repair strategies need to be applied in bacteria and archaea living in those extreme circumstances.
It is a difficult task to evaluate the results obtained from mostly unknown species. Therefore we applied again the HMMER profiling: for the identified DNA repair genes in the extreme metagenomes, we prepared new HMMER profiles (for each genes separately, subsequent to a cluster analysis); and we searched for similarities to those profiles in well-known model organisms.
We have found well known DNA repair proteins, lots of proteins with unknown functions, and also proteins with known, but different functions in the model organisms. We describe the results of this work in our presentation.

SW01.S3–43
New players in recognition of AP sites in clustered DNA damages
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DNA repair is the central mechanism to keep integrity of cell genome. One of the most abundant lesions in DNA is apurinic/apyrimidinic sites (AP) sites arising spontaneously or under genotoxic stress conditions. AP sites are unstable and cytotoxic. Attempted repair of bistranded AP sites (i.e. situated in both DNA chains) can result in formation of double-strand breaks – the most deleterious DNA lesion. One can suggest an existence of proteins that are able to specifically interact with AP sites in clustered DNA damages and regulate their processing.
DNA containing bistranded AP sites were used to trap in mammalian cell extracts proteins interacting with AP sites via Schiff-base intermediate.
Cross-linked proteins were identified by peptide mass mapping (based on MALDI-TOF-MS analysis) and immunochemical approaches.
High-mobility group box 1 and 2 proteins, abundant multi-functional non-histone chromatin proteins, were shown to more efficiently interact with bistranded AP sites than isolated ones, and in reconstituted system interfere with AP sites hydrolysis by AP endonuclease 1, the main mammalian protein cleaving AP sites.
Unexpectedly, AP endonuclease 1 was also identified as a target of cross-linking to bistranded AP sites via Schiff-base-mediated mechanism without a concomitant cleavage of AP sites. Efficiency of bistranded AP site hydrolysis by AP endonuclease 1 was evaluated.

Influence of base excision repair proteins – poly(ADP-ribose) polymerases 1 and 2 – on processing of clustered AP sites was studied in reconstituted system.

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SW01.S3–44
Double strand breaks introduced by phage T4 homing endonuclease SegD is repaired by alternative mechanism
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Homing endonucleases are a class of site-specific endonucleases, which are encoded by mobile genetic elements – introns of group I, inteins or freestanding ORFs. These enzymes initiate nonreciprocal transfer of DNA segments containing their own genes and the flanking sequences by introducing double-strand breaks (DSB) in to the homologous allele lacking homing endonuclease gene. DSB repair process proceeds by the mechanism of homologous recombination using intact DNA of the homologous molecule containing the endonuclease ORF as the donor. The transfer of nuclease gene and the flanking sequences in to the new allele is accompanied by a distortion of the cleavage site of homing endonuclease. This distortion serves as a protective mechanism that prevents the genome from deleterious lesions.

Bacteriophage T4 SegD protein belongs to a family of GIY-YIG homing endonucleases and encoded by freestanding gene. ORF of segD is located between structural genes 23 and 24 and oriented oppositely with respect to these genes. We have sequenced the gene 23–24 regions of T4-related bacteriophages and found that 11 of them lack segD ORF. We have determined the cleavage site of SegD endonuclease, which is located at the 3′-end of the gene 23 in 52 nt from its own ORF. The properties of SegD were significantly different from other T4 Seg-endonucleases: it cleaved a DNA with the formation of 3′-overhangs of 3 nt long; the complex of SegD with DNA substrate was not stable in vitro, and SegD cleavage site remained intact in the genome of phages T4, T6, RB55 and RB59, harbouring the endonuclease gene. We found that segD gene was expressed in the course of T4 infection, and the cytosine modifications of T4 DNA did not affect SegD endonuclease activity. Nevertheless, the homing of segD gene was not observed in the crosses of phage T4 with T2L, which lacks segD ORF. A model system where SegD site was inserted into rIIIB gene of phage T4 was constructed and SegD-dependent genetic recombination in the rII region was studied. In the crosses, where only one of the parents contained SegD site in rIIIB, the recombiant frequencies on SegD+ and SegD− background were the same. Whereas, when both parent phages contained SegD site in rIIIB, the recombiant frequency on SegD+ background was 2.4 times higher than in the crosses of segD phages. The results of genetic analysis indicated that repair of SegD-induced breaks did not proceed by homologous recombination. Based on our data we suggest that SegD-induced DSB is repaired by the alternative mechanism through the homologous ends joining.

SW01.S3–45
Stopped-flow kinetic analysis of the role of Asn212 in the catalytic mechanism of human AP endonuclease 1
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Human AP endonuclease 1 (APE1) is a crucial enzyme of DNA base excision repair which incises the phosphodiester backbone 5′ to the abasic site. The resulting 3′-OH moiety is recognized and processed by DNA polymerase β. Crystallographic data show that the side chain of Asn 212 in the APE1 active site participates in DNA substrate stabilization through hydrogen bonding to the scissile phosphate moiety. Previous studies have shown an importance of Asn212 for catalytic activity of the enzyme. To investigate the role of Asn 212 at particular steps of the reaction mechanism we used two APE1 mutants (N212D and N212A) in stopped-flow kinetic analysis combined with fluorescence detection. The 12 bp oligonucleotide duplexes containing natural AP site or its tetrahydrofuran (F) analogue were used as specific substrates for APE1. Time courses of conformational changes of the protein molecule were obtained by detecting of intrinsic Trp fluorescence. To observe conformational transitions in abasic DNA the fluorescent analogue of adenine, 2-aminopurine, was introduced into the substrates 3′ to the site of damage. The series of stopped-flow traces obtained under single-turnover conditions demonstrated multiple conformational transitions of the enzyme–substrate complex along the reaction coordinate. Based on the quantitative analysis of fluorescent data we have proposed the kinetic mechanism and have determined the values of rate constants. The results have shown a 10 times decrease of the rate of binding steps and approximately three orders of magnitude decrease of the incision rate for both mutant forms as compared to WT APE1. In addition, in the case of alanine substitution the rate of cleaved product accumulation approached the rate of enzyme inactivation making difficult analysis of the data. Based on the findings, we propose the Asn-212 residue of APE1 to be more essential for effective catalysis than for specific binding of DNA substrates.

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SW01.S3–46
Human 8-oxoguanine DNA glycosylase C253I and C253L mutant forms in the DNA repair process
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DNA damage constantly occurs in living cells due to environmental factors and normal metabolic processes. 8-Oxoguanine-
DNA glycosylase hOgg1 is one of the most important human DNA repair enzyme that removes 8-oxoguanine (oxoGua), a pre-mutagenic oxidative base lesion, from DNA. Amino acid Cys-253 of hOgg1 molecule participates in 8-oxoguanine coordination after its extrusion from DNA helix to the active site. In order to clarify the role of this base coordination and to reveal its place in the overall enzymatic process we have constructed mutants replacing the Cys253 residue with either isoleucine or leucine. The stopped-flow kinetic study in a combination with fluorescent detection was used in order to reveal conformational dynamics during protein-DNA interaction. Changes in the protein-DNA complex structure were followed by registration of the fluorescence intensity of Trp residues and by FRET (Fluorescence Resonance Energy Transfer) using Cy3/Cy5 labels in DNA substrates. Alterations of fluorescence signals indicate sequential conformational transitions in macromolecules during the catalytic cycle. It was shown that although Cys-253 mutations distort the active site of hOgg1 and greatly decreases the catalytic proficiency of the enzyme, they do not fully prevent 8-oxoguanine and AP-site damages recognition, sampling and excision. For both mutant forms, a notable increase in the nicked product formation rates was observed in a presence of 8-bromoguanine (BrGua) during interaction with both AP- and oxoGua-substrates. Of note, the β-elimination rate did not depend on the order of mixing. This may indicate that BrGua binding is not tight enough to prevent subsequent entrance and co-ordination of oxoGua-substrate.

Results obtained for C253I and C253L forms of hOgg1 resonate well with the concept of active site plasticity of enzymes. They indicate that the active site is flexible enough to compensate partially distortions caused by inappropriate amino acid residues.

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SW01.S3–47
R56-sensitising effect of DNA-repair inhibitors in normal and cancer cells

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Double strand breaks (DSB) are considered to be one of the most lethal damage in DNA induced by ionizing radiation (IR). These lesions activate DNA damage response pathway. Two main repair pathways are non-homologous end joining (NHEJ) and homologous recombination (HR) where the PI3-K related kinases: DNA-dependent protein kinase (DNA-PK), ataxia telangietasia mutated (ATM) and ATM- and Rad3-related (ATR) are pivotal kinases and phosphorylate a large number of proteins involved in cell proliferation, cell cycle arrest, cell survival and cell death. ATM and DNA-PK are activated by DSB whereas ATR is activated predominantly by single strand breaks arising during DSB formation. ATM is the main kinase of HR contrary to DNA-PK which has a crucial role in NHEJ.

An effort to inhibit DNA damage response in tumor cells in order to prevent DSB repair is promising therapeutic strategy for cancer treatment. Majority of tumors treated by IR or chemotherapeutics are resistant against this anti-cancer therapy and the effect of this treatment is considerably low because of enhancement of DNA repair processes in tumor cells. Inhibitors, which can increase cytotoxic effects due to inhibition of DNA repair were developed and are investigated as potential radio and chemo-sensitizers.

In the presented study we describe the effect of inhibition of three pivotal DNA repair kinases on molecular mechanisms triggered by IR. We employed NU7441 (1 μM), a specific inhibitor of DNA-PK; KU55933 (10 μM), a specific inhibitor of ATM, and VE-821 (10 μM), a specific inhibitor of ATR and we studied the effects of pre-treatment with inhibitors prior irradiation of normal human lung fibroblasts (NHLF, p53-wildtype) in comparison to osteosarcoma cell line (SAOS-2, p53-negative). Our data obtained by fluorescence microscopy and immunodetection techniques evaluate the radio-sensitizing effect of the given inhibitors. The importance of individual kinases involved in radio-sensitization of normal and cancer cells will be discussed in the context of p53-status.

SW01.S3–48
Tp2 interacts with and regulates NPM expression levels under genotoxic stress

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Tp2 is a MAP3 kinase which participates in signal transduction cascades triggered during immunological responses against various pathogens or their products. Recent evidence suggests that Tp2 is also a physiological regulator of tumorigenesis as ablation of the Tp2 gene in mice results in enhanced susceptibility to various types of cancer. Nucleophosmin (B23) is a predominant nucleolar phosphoprotein that shuttles between nucleolus-nucleus and cytoplasm. It acts as a molecular chaperone that drives ribogenesis, maintains genomic stability and regulates chromatin conformation. It is also a key component of DNA-damage response pathways. Mutants and chimeric forms of NPM have been found to be implicated in various blood malignancies, while its abnormal expression or localization often serve as prognostic markers for cancer. High-throughput proteomics identified NPM as a putative interactor of Tp2. Herein we confirm the structural and functional relationship between the two proteins. We show that whereas Tp2 is predominantly cytosolic, it also localizes in the nucleoli and associates with NPM. The downregulation or absence of Tp2 ameliorates NPM induction following exposure to various genotoxic factors. Tp2 downregulation blocks NPM phosphorylation at T199, which associates with NPM ubiquitination and proteasomal degradation. Deregulation of NPM in the absence of Tp2 attenuates NPM-p53 interaction and alters transcriptional activation of p53 and cell cycle progression.

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SW01.S3–49
Induction of DNA damage in A549 lung adenocarcinoma cells by inhibitors of type I and II topoisomerase

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DNA topoisomerase I (Top1) and topoisomerase II (Top2) inhibitors are widely used to treat a variety of cancers. Their mechanism of action involves stabilization of otherwise transient (‘cleavable’) complexes between Top1 or Top2 and DNA; collisions of DNA replication forks with such stabilized complexes lead to formation of DNA double-strand breaks (DSBs).

We directly assessed the relationship between induction of DNA Damage Response (revealed as γH2AX foci, and DNA Damage Repair marked as Rad51 foci (HR pathway)) and DNA replication (detected using 5-ethyl-2′-deoxyuridine (EdU) as a DNA precursor), in A549 cells treated with Top1 inhibitor – camptothecin (Cpt) or Top2 inhibitor – etoposide (Etp). Multiparameter laser scanning cytometry analysis of cells exposed to Cpt revealed that only replicating cells show induction of γH2AX and a strong correlation between formation of DSBs and DNA replication. In cells treated with Etp, the correlation was weaker; moreover Etp caused induction of γH2AX in non-replicating cells. Confocal imaging of nuclei of cells treated with Cpt revealed the presence of γH2AX/Rad51 foci predominantly in DNA of replicating cells and close association of γH2AX/Rad51 foci with DNA replication sites. In cells treated with Etp, the γH2AX foci were induced in DNA replicating as well as non-replicating cells but a close association between a large proportion of γH2AX/Rad51 foci and DNA replication sites was still apparent (Rybak et al.; Cytometry A, in press).

These data are consistent with the view that collision of DNA replication forks with cleavable Top1–DNA complexes stabilized by Cpt is the sole cause of induction of DSBs. Additional mechanisms such as involvement of transcription and/or generation of oxidative stress may contribute to DSBs induction by Etp. Combining laser scanning cytometry with confocal microscopy in studies of DNA damage signalling and replication stress constitutes a new approach to mechanistic studies of a relationship between DNA replication and induction of DNA damage.

SW01.S3–50
Quantitative imaging analysis of replication stress in cells exposed to DNA targeting anticancer drugs and oxidative stress

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Background: A recently described method (Berniak et al., submitted) of quantitative analysis of spatial (3D) relationship between discrete nuclear events detected by confocal microscopy was applied in analysis of a dependence between sites of DNA damage signaling (γH2AX foci) and DNA replication (EdU incorporation) in cells subjected to treatment with topoisomerase inhibitors camptothecin (CPT), etoposide (ETP), mitoxantrone (MTX), cis-platinum (CS) or hydrogen peroxide (H2O2).

Methods: Newly synthesized DNA was fluorescently labeled using a precursor EdU (5-ethyl-2′-deoxyuridine) and a ‘click’ reaction. γH2AX foci were labeled by immunofluorescence. Images of replication and histone H2AX phosphorylation sites were recorded using confocal microscopy aided with deconvolution. Nearest-neighbor and correlation analyses were performed using an algorithm written specifically for this task.

Results: CPT induces γH2AX foci, likely reporting formation of double-strand DNA breaks (DSBs), almost exclusively at sites of DNA replication. ETP induces γH2AX in S-phase, with a moderate tendency toward replication sites. MTX induces γH2AX foci with no detectable preference for replication foci. Etoposide (ETP) phosphorylation induced by CS is detected 2 and 4 h after exposure to the drug and largely coincides with replication foci. Oxidative stress leads to induction of γH2AX in replicating as well as non-replicating cells, and only a weak tendency toward damage at sites of DNA replication.

Conclusions: High degree of colocalization of EdU and γH2AX sites in cells treated with CPT is coherent with the known mechanism of induction of DSBs by DNA topoisomerase I (topo1) inhibitors at sites of collision of moving replication forks with topo1-DNA ‘cleavable complexes’ stabilized by CPT. The moderate or poor correlation of replication sites with γH2AX as seen in the case of ETP or MX (also observed with these drugs in non-replicating G1 and G2 cells), indicates on the mechanism of DNA damage unrelated to replication. The increased number of replication foci observed in cells treated with CPT suggests that stalling replicating forks may trigger activation of new DNA replication origins. This agrees with a postulated plasticity of replication origins.

SW01.S3–51
Xrc1 recruitment to endogenous DNA damage in replicating cells

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Background: Xrc1 (X-ray repair cross-complementing protein 1) is a DNA repair factor involved in Base Excision Repair (BER) pathway. It takes part in repair of DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents. Xrc1 protein interacts with DNA ligase III, polymerase beta and poly (ADP-ribose) polymerase (PARP) and is a protein loading platform.

Goal: This work was focused on investigating a relationship between spontaneous induction of single-strand DNA breaks, DNA replication and formation of microfoci containing Xrc1.

Methods: Fluorescence live cell confocal imaging of pmRFP-Xrc1 and eGFP-PCNA fusion proteins, and quantitative analysis of 3D data stacks and time-lapse sequences were used. DNA replication was also detected by incorporation of EdU (5-ethyl-2′-deoxyuridine) followed by ‘click’ reaction, imaged in fixed cells; γH2AX, as a marker of double-strand DNA breaks, was detected by immunofluorescence.

Results: In a population of transfected HeLa cells maintained in an in vitro culture a small proportion of cells develop spontaneously conspicuous Xrc1 microfoci. In the scrutinized cases the foci are induced in S-phase and their number grows in time. The Xrc1 foci are found almost exclusively in the immediate vicinity of replicating DNA. Time-lapse recordings demonstrate that, following the formation of Xrc1 foci, replication is resumed close to these foci. Most cells exhibiting the numerous Xrc1 foci adjac-
cent to replication regions eventually die, although some of these cells apparently survive the replication stress, complete replication, enter G2 phase and divide successfully.

Conclusions: Live cell imaging and immunofluorescence experiments suggest that spontaneously formed single-strand DNA breaks may be associated with replication forks. Although most cells that developed a high number of single-strand DNA breaks in S-phase are destined to die, apparently some are capable of repairing the damage and returning to the cell cycle.

SW01.S3–52
Heterochromatin protein 1beta – a key factor in DNA repair and replication
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Background: Heterochromatin protein 1 (HP1) is a small non-histone chromosomal protein, originally described as major component of heterochromatin and an epigenetic regulator. However, HP1 is emerging now as a multifunctional protein involved in several key nuclear processes. We have demonstrated that HP1\(a\) and HP1\(\beta\) are recruited to sites of oxidative DNA damage (1, 2, 3); recruitment to other types of damage has also been demonstrated (4).

Methods: Recruitment and dynamics of HP1\(\beta\) was studied using live cell imaging, fluorescence recovery after photobleaching (FRAP), fluorescence resonance energy transfer detected by lifetime microscopy (FLIM/FRET) and bimolecular fluorescence complementation assay (BIFC). Local DNA damage was induced by low intensity visible light, in the absence of exogenous photo-sensitizers (5).

Results and Discussion: We demonstrate that HP1\(\beta\) forms a complex with Proliferating Cell Nuclear Antigen (PCNA), a key factor involved in DNA replication and repair. The HP1-PCNA complex is engaged in DNA replication; it is also recruited to ss and ds DNA breaks (6). FRET-FLIM live cell studies provide evidence of association and close proximity between HP1\(\beta\) and PCNA in the complex. FRAP demonstrates that HP1\(\beta\)-PCNA complexes are highly mobile in nonreplicating nuclei, but when engaged in DNA replication, within DNA replication foci, they are bound and do not exchange with the mobile pool (7). HP1\(\beta\), which is associated with PCNA in the regions of DNA repair, is bound and does not exchange with the mobile pool, suggesting that HP1\(\beta\) in association with PCNA may be a component of a DNA repair complex. The role of HP1 in DNA repair and replication remains unknown; we postulate that HP1\(\beta\) may act as a loading platform which recruits various factors involved in DNA repair and replication.

References
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SW01.S3–53
Modification of UVC-induced DNA destruction in vitro
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UVC-irradiation produces several destructions in DNA. They can be revealed in a model water solution by the methods of UV spectroscopy, circular dichroism, low-gradient viscosity, LS/MS. In the present work we observed UVC-dose dependent changes in the DNA spectral properties, degree of helicity, nitrogenous bases amount and the specific volume of the macromolecule. We studied the influence of ionic conditions and several natural antioxidants (caffeine, catechin) on the UVC-light action on DNA structure. It was found that radiation efficiency reduces with the increase in low-molecular electrolyte concentration, as well as in the presence of the examined antioxidants in the exposed DNA solution.

SW01.S3–54
The detection of nucleotide excision repair activity: new perspectives
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The DNA nucleotide excision repair (NER) system recognizes and removes from DNA a wide variety of structurally diverse helix-distorting bulky adducts. One of the advanced and upcoming approaches to DNA repair investigation is based on the application of synthetic DNA molecules, containing lesion-simulating adducts. The characterization of specific excision activities in cell extract and reconstituted systems is a significant problem and could represent a major concern for fundamental research and for medical applications. An effective standard substrate is necessary to enable the studies of a key stage of NER, the elimination of the damaged DNA fragment. Elaboration of such substrate will permit to bring a further insight into the mechanisms of NER, its role in the development of degenerative diseases, and to investigate the effects of gene-environment interactions on DNA repair. So the aim of the present study was to design the new effective method for detection of damaged strand excision in the nucleotide excision repair.

The optimal strategy of model DNA synthesis was elaborated. The solid phase synthesis using bulky modified non-nucleoside phosphoamidites was suggested as most straightforward approach for creation of model DNA independently of the length and nucleotide sequence. Taking into account the knowledge of the NER recognizable DNA lesions the fluoresein and antracene derivatives have been chosen as bulky substituent. Also, original in vitro NER assay was improved to protect the products of specific excision from nonspecific degradation.

Good substrate properties of obtained model DNAs in conjunction with improved in vitro NER assay significantly increase the sensitivity of NER activity detection method. This permits to use fluorescent label for in vitro NER assay instead of radioactive label and open a new perspectives for the detection of nucleotide excision repair activity in fundamental and medical research.

This work was supported by RFBR (project 12-04-00487) and the Russian Ministry of Education and Science (contract 14.B37.21.0188).
The major human apurinic/apyrimidinic endonuclease 1 (APE1) is a key enzyme in the base excision repair (BER) and nucleotide incision repair (NIR) pathways. BER is initiated by DNA glycosylases, excising the damaged and/or mispaired bases to produce apurinic/apyrimidinic sites (AP sites). AP sites are generated also through the spontaneous loss of bases (mainly purines). During BER pathway DNA in human cells is hydrolytically nicked 5' to the AP site by APE1. Repair of certain base lesions can be initiated directly by the AP endonucleases alone in NIR, by-passing the DNA glycosylase step. During this process, an AP endonuclease introduces a nick 5' to the damaged deoxynucleotide, generating a 3'-hydroxyl terminus and a 5'-phosphate terminus. Thus, the NIR pathway avoids the formation of potentially toxic AP-intermediates. Using a stopped-flow fluorescence method we analyzed the conformational dynamics and kinetic mechanism of wild-type APE1 and its mutants APE1K98A and NΔ61APE1. DNA substrates used in this study contained AP site, tetrahydrofuran, 5,6-dihydouridine (DHU) or α-2-deoxyadenosine. Our data suggest that APE1 can pre-exist in two conformations and that the conformational selection and induced fit occur during the enzyme action. The enzyme release from the complex with the nicked DNA product limits the overall NIR process and determines its rate in the steady-state conditions. The comparison of the kinetic constants of mutants to those of wtAPE1 provides us with a view of the roles of lysine-98 and of REF1 domain. We have shown that during both BER and NIR pathways Lys98 is important in the 5'-phosphodiester bond hydrolysis of DNA substrate. This amino acid substitution influences the catalysis in NIR more extensively, than in BER pathway. The REF1 is required for the 5'-phosphodiester bond hydrolysis in NIR, but not in BER pathway. Our data reveal that APE1 uses the same active site to catalyze the cleavage of DHU- and AP-substrates. The protein is probably able to form different conformations in the region of the active site, which are responsible for the incision of such structurally unrelated lesions as AP site and DHU.

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Metabolome and proteome analysis of *E. coli* lacking HU protein

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The nucleoid-associated protein HU plays an important role in bacterial nucleoid organization and is involved in numerous processes including transposition, recombination and DNA repair. *E. coli* lacking HU protein are viable while grow slowly and are sensitive to a variety of stresses.

In this work we identified the proteins that are differentially expressed in wild-type *E. coli* and in *E. coli* lacking HU protein by 2D gel-electrophoresis with fluorescent staining followed by the protein MALDI identification. To complement the proteome study we identified as many intracellular metabolites as possible in wild-type *E. coli* and in *E. coli* lacking HU protein by flow injection time-of-flight mass spectrometry. Metabolite identity was confirmed by fragmentation of previously detected ions by target mass spectrometry. The selected liquid chromatography approach, hydrophilic interaction chromatography with amino and silica columns, effectively separates highly polar cellular metabolites prior to their detection on a high accuracy mass spectrometer in positive and negative acquisition mode for each column. Here we present reliable measurement of a hundred metabolites, and discuss the differences in metabolite profile between wild-type and *huAB- E. coli*. We compare the differences in metabolome profile with the differences in protein expression caused by the lack of HU.
Recognition of DNA damages by human 8-oxoguanine DNA glycosylase

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Extensive structural studies of human DNA glycosylase hOGG1 have revealed the substantial conformational changes of enzyme molecule; however, at present there is little information about the time scale of rearrangements of protein structure as well as dynamic behavior of individual amino acids. Here we examined the role of certain catalytically important amino acids in hOGG1 enzymatic pathway and described their involvement in the step-by-step mechanism of oxidative DNA lesion recognition. The conformational dynamics of hOGG1 wild-type and mutants Y203W, Y203A, H270W, F45W, F319W and K249Q and DNA-substrates were investigated by the fluorescence stopped-flow method.

The analysis of kinetic data obtained in this study significantly improves understanding of the step-by-step molecular mechanism of hOGG1 lesion recognition process. Our data showed that the function of Tyr-203 residue is not only to conserve of the kinked state of DNA duplex but to serve as ‘lesion-sensor needle’ in the discrimination between normal and damaged bases. Also we demonstrate the role of Lys-249 plays an important role in the early step of the damaged nucleotide binding and flipping out process. The suggestion that Asp-268 residue is responsible for hydrolysis of N-glycosidic bond, whereas Lys-249 is a key amino acid in sugar-phosphate bond cleavage (β-elimination reaction) was confirmed. The role of His-270 in the xoOGF flipping out process was identified.

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Development of ribonuclease H2A inhibitors as anticancer agents

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Introduction: While patients with advanced cancers may initially respond to chemotherapy, they are rarely cured and most of them ultimately relapse and die [1]. As the effects of chemotherapy and radiation on cancer cells likely occur through induction of DNA damage, resistance to genotoxic agents in advanced cancer might be associated with factors governing DNA break repair, chromatin structure and genome stability. Previously, we and others had identified ribonucleases H as factors strongly associated with both increased chromosomal breaks and loss [2]. Recent evidence has demonstrated RNASEH2A overexpression in different solid cancers [3]. Furthermore, latest bioinformatic studies determined RNASEH2A among the top 2% of targets for cancer drug development. To this end we aimed to develop RNASEH2A inhibitors.

Methods: We utilized a fluorescence resonance energy transfer assay for high-throughput screening of inhibitors of RNASEH2A activity. More than 2000 compounds from NCI chemical libraries were screened with this assay. The Ki’s of each inhibitor were determined by using the GraphPad Prism Program. In order to assess the role of RNASEH2A in tumor survival we treated several cancer cell lines with different levels of RNASEH2A expression (MV-4-11, HL-60, HeLa) with increasing concentrations of several RNASEH2A inhibitors for 0, 4, 24, 48 and 72 h. The effects on cell viability, apoptosis and cell cycle were assessed by using standard assays.

Results: About 70 inhibitors were uncovered, most of which show relatively high potency and high specificity. RNASEH2A inhibitors affected the viability of cell lines with high levels of RNASEH2A expression. No effect was observed in HeLa cell line with low or no RNASEH2A expression.

References:

Molecular dissection of the methylome of Burkholderia cenocepacia J2315

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B. cenocepacia is a pathogenic gram-negative bacterium that often causes an opportunistic infection in cystic fibrosis patients. It is highly antibiotic-resistant, transmissible, and often lethal. Although genome sequences of several strains are available, the study of this organism, like many pathogens, has been relatively limited, and genetic study is difficult. The aim of this work was to analyze the genomic DNA methylation patterns from the sequenced strain, J2315, and identify specificities of putative DNA methyltransferases. This might facilitate the development of an efficient transformation system to enhance genetic studies of this strain. We took advantage of the recently developed platform for single-molecule real time sequencing by Pacific Biosciences. This next generation sequencing technology allowed us not only to perform high throughput DNA sequencing, but also to identify the epigenetic status of the DNA using the polymerase’s kinetic signature on the template during the reaction. The kinetic signature analysis of B. cenocepacia J2315 genomic DNA revealed three modified motifs. Additionally, a number of ORF’s encoding putative DNA methyltransferases were cloned into plasmid vectors, and their specificities were determined using SMRT sequencing.

A Type III restriction-modification system called M.BceJ resulted in a modification in the recognition sequence CA-CAG. 98.4% of all CACAG sequences in the genome were modified on just one strand as indicated, which is typical of a Type III methyltransferase. Cloning ORF BCAL3494 showed it to be the gene responsible. The genomic motif GTWWAC, containing
One rather interesting methyltransferase is encoded by ORF pBCA072, which is located on a plasmid. It results in single-stranded, non-specific \(^{m6}A\) modification, but was only detected very difficult. In *E. coli* indicating that the system is probably inactive in this strain.

One other interesting methyltransferase is encoded by ORF pBCA072, which is located on a plasmid. It results in single-stranded, non-specific \(^{m6}A\) modification, but was only detected on plasmid DNA. We have called this enzyme M.BceJII. A similar enzyme has been found on an *E. coli* plasmid and both could possibly play a restricted role during DNA replication.

The M and S subunits of a putative Type I restriction-modification system BcJRF418P have been cloned, but no modifications were detected either on genomic or plasmid DNA indicating that the system is probably inactive in this strain.

**SW01.W4 Evolutionary Genomics (I-W4)**

**SW01.W4–1**

**HGP and -omics: Big Science and Big Data**

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2013 will see the global celebrations for the 60th anniversary of discovery of DNA double helix and 10th anniversary of the official completion of the International Human Genome Project (HGP). The HGP has “changed biology and biotech for every” by cultivating a new science, GENOMICS, and could be regarded as the first practice in its real sense. The impact of the HGP on biology might be “digitalization” and “-omesization”, characterized by “big’s”, “big” data, “big” science, “big” platform, and “big” collaboration.

The soul and pillars of genomics are based on two believes in life, i.e. “life is of sequence (J. Watson)” and “life is digital (J. Suston)”. The past few years have seen the combined explosion in the amount of sequence and other “-omics” data sets and the demands for computing capacity. “The 1K Genomes Project”, together with other ongoing human genomes projects internationally, have generated data amount which was unimaginable before. With the rapid development of digitalized “Tree of Life” on the globe, even more data will be obtained from the other animals and plants, especially the enormous newly discovered microbes by metagenomics approach. No doubt, all this creates new challenges of scale for computation, storage, global transfer, and interpretation of petascale data. Generally speaking, two models, centralized and decentralized, will be both on operation parallelly, and cloud computing has the potential to be of help. Besides the technical challenges, the data safety, privacy and other bioethical issues, would be added to the challenges.

**SW01.W4–2**

**Sequencing ancient and really ancient genomes illuminates horse evolution**

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The domestication of the Horse has played a critical role in human societies, facilitating the exploration of novel territories and providing military advantage in warfare, supporting the expansion of continental-scale nomad empires of the Hittites, Huns and Mongols. Mounted riding also helped controlling larger herds, which likely contributed to increase domestic animal productivity and thereby resulted in economical and urban development. Although still debated, Indo-European languages, whose speakers represent almost half the population worldwide, may possibly have spread on horseback some 5.5 ka BP. Today, almost all horses have become extinct in the wild, with the exception of the endangered, inbred and possibly admixed Przewalski’s horse, precluding direct comparison of wild and domestic genomes. Ancient DNA approaches aiming however no go back in time before horses were domesticated and deliver genome sequences of pre-domesticated horses. We use state-of-the-art ancient DNA methods in combination with second and third generation sequencing technologies in order to reconstruct complete genomes of a series of such ancient genomes. Comparisons to modern genomes from a handful of domestic breeds promises to reveal which genomic features have been selected early in the process of horse domestication and have supported this major revolution in human history. In addition to revealing domestication-related traits, ancient horse genomes will allow us to dig out the deep evolutionary past of the horse lineage, to reconstruct the long-term demographic trajectory of horse populations during past major climatic shifts as well as to track in almost real time the dynamic changes of selective forces and the expansion of major gene families.

**SW01.W4–3**

**Sequencing the Human genome as a tool for refinement of some anthropological and historical hypotheses**

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The study of human genetic variation within a geographical context provides insight into how selection, historical events, and migrations contribute to modern human populations. Genome-wide population studies have sampled human genetic diversity across much of the globe, but have largely left out populations in Northern Eurasia. We have conducted a high-resolution population genomic analysis of Northern Eurasia, a landmass unique in its geographic and ethnic scope. Genotype data were assembled from 5,764 people worldwide, including 923 newly collected samples representing 29 distinct ethnic groups ranging from Central Europe to the Pacific coast. Principal component analysis using 200,000 single-nucleotide polymorphisms (SNPs) effectively separated the populations into a two-dimensional map of the Russian Federation. As few as 17 SNPs were sufficient to position an individual in the East-West dimension. Strikingly, we found no admixture signatures between present-day Russian and Tatar populations, despite centuries of cohabitation. Rather, modern Russians appeared highly similar to other Slavic groups, but harbored some genetic signatures of Finno-Ugric groups. A genome-wide FST pairwise matrix provided diversity estimates within and among the Northern Eurasian populations. Finally, extended
haplotype homozygosity analysis identified loci under natural selection, with the strongest signal arising from the Myoferlin (MYOF) gene. The present data add a final piece to the global puzzle of human genetic diversity, and shed light on the effects of natural selection and human history in populations of Northern Eurasia. Our findings will promote the design of effective genome-wide association studies, forensics, and parenthesis tests involving individuals of Eurasian descent.

**SW01.W4–4**

Global hypomethylation and promoter related demethylation are associated with copy number loss of DNMT1 gene and unfavourable clinical outcome in primary melanomas

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In melanoma, the most aggressive form of skin neoplasm, the presence of transposon related hypomethylation was reported previously along with hypermethylation which is usually localized into the transcriptionally active gene parts. However, no methylation-based distinction has been drawn among diverse melanomas with different clinical behaviours.

Our goal was to persuade a study for obtaining better insights into how distinct types of DNA methylation changes associated with melanoma progression. As Knudson’s two-hit hypothesis is often achieved through a combination of differing types of genomic alterations, we also aimed to investigate whether methylation patterns are associated with other types of somatic alterations such as the most frequent mutation and DNA copy number alteration.

We found that transposonal hypomethylation was associated with shortened relapse free survival of melanoma patients, however, Cox regression pointed direct relationship of the overall loss of copy number of DNMT1 promoters in primary melanomas. We concluded that hypermethylation pattern of melanoma is a part of an integrated apparatus of somatic DNA alterations. As DNMT1 deletion often occurs at late stages, it can contribute to decreased promoter methylation levels observed in samples with unfavourable clinical outcome. TÁMOP-4.2.2/B-10/1-2010-0024 and TÁMOP 4.2.2.A–11/1/KONV-2012-0031.

**SW01.W4–5**

Polymorphisms in Her2/Neu and MMP1 genes and associations with breast cancer risk

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Metastasis is responsible for the majority of breast cancer related to mortality. Matrix metalloproteinases (MMPs) are proteolytic enzymes that destruct the extracellular matrix. They play an important role in tumor invasion and metastasis. MMP1 is responsible for the destruction of collagen. Cancer patients with poor prognosis have MMP1 overexpression. Her2/neu is a protooncogene that encodes a transmembrane glycoprotein with tyrosine kinase activity. Overexpression and/or amplification of Her2/neu is associated with poor prognosis in breast cancers. In this study we aimed to investigate the possible association between breast cancer and the Her2/neu or MMP1 polymorphisms, whether breast cancer is more aggressive in the patients with both polymorphisms, and the effects of these genes on the pathogenesis of the disease. In this study 90 breast cancer and in 95 healthy subjects were included. In order to detect the SNPs on MMP1 promoter region (2G/G) and on HER2 gene (Val655Le), PCR and RFLP were performed. The statistical analyses were performed to the results. There was no association with the genotypes of MMP1 gene (2G/2G, 2G/G, G/G) and the other parameters (tumor size, histological grade, axillary lymph node involvement, stromal reaction, tumor type, ER, PR, in situ component) of disease in the patients. However, when the 2G/2G and 1G/2G genotypes were evaluated together, significant association (p < 0.05) was detected with the lack of progesterone receptors in the patient group. There was significant relation between histological grade and the Her2/Neu I/V + V/V genotypes in patients. The patients (66.7%) with both MMP1 2G/G genotype and Her2/neu I/V genotype had invasive ductal carcinoma. We concluded that the MMP1 2G/G and HER2 Val655Le polymorphisms can not be risk factors for breast cancer alone, however they may be considered as a possible risk factor for the invasive tumor type in patients who have the type of two polymorphisms coincidentally, and that it would be appropriate to increase the number of subjects in the study group for more accurate decision on the results.

**SW01.W4–6**

Increased transcriptional activity of CD40LG in patients with essential hypertension

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Essential hypertension is a common disorder characterized by chronic increase of blood pressure in absence of any specific condition that may cause its elevation. A number of studies have demonstrated that inflammation is implicated in the development of hypertension, but the exact mechanism remains unclear. CD40-CD40LG interaction plays an important role in inflammation process, as well as in matrix degradation, thrombosis and vasoconstriction.

The objective of our study was to investigate transcriptional activity and genetic variance of CD40LG gene in patients with essential hypertension and healthy subjects.

We studied expression profiles of CD40LG gene in peripheral blood leucocytes of 20 patients with essential hypertension and 20 control subjects, and found that transcriptional activity of
**CD40LG** gene was increased in hypertensive patients (FC, fold change, 13.36). To validate the obtained result, we performed quantitative real-time RT-PCR in 24 patients with essential hypertension and 17 healthy individuals, which has shown that relative **CD40LG** mRNA levels were significantly increased in patients with essential hypertension compared to the control group (7.64 ± 2.27 versus 0.96 ± 0.08, p < 0.001). To further investigate the link between **CD40LG** gene and hypertension, we genotyped **CD40LG** rs715762 polymorphic variant in the group of 217 patients with essential hypertension and 242 healthy control subjects, but found no association with hypertension. Moreover, no correlation was found between **CD40LG** rs715762 and **CD40LG** expression patterns, suggesting that said genetic variant does not have an impact on the transcriptional activity of the gene.

In conclusion, our results are consistent with the hypothesis of inflammatory basis of hypertension, but further investigation is needed to understand the role of certain molecular pathways of the disease.

**SW01.W4–7**

The IL6 rs1800795 polymorphism (−174G/C) relationship to metabolic indices in Bulgarian sample

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**Introduction:** Nowadays obesity is a serious burden all over the world. Low-grade inflammation is believed to accompany obesity. Some studies have associated the G allele of the IL-6 gene with obesity, and related metabolic disorders. On the opposite, other publications report that C allele carriers of this gene are predisposed to such metabolic disturbances.

**Aim of Study:** The present study aims to investigate allele frequencies of rs1800795 polymorphism in the IL6 gene and its relation to markers of metabolic disturbances in the Bulgarian population.

**Study Subjects and Methods:** The study included 182 Bulgarian volunteers ≥18 years of age. BMI, levels of plasma glucose, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL), blood pressure (systolic/diastolic) and waist and hip circumferences) were measured and related to the IL6 SNP genotype. Genomic DNA isolated from whole blood was genotyped by S'-exonuclease assay using Real-time PCR 7500.

**Results:** The established genotype distribution for the Bulgarian sample was similar to other Caucasian populations. However, GG carriers exhibited higher plasma glucose levels than the G/C and C/C carriers (5.09 ± 0.19 versus 4.77 ± 0.10 mM; p = 0.04). CC carriers had lower, although not significantly, levels of TAG and total cholesterol as compared to GG and GC carriers. Within the obese group individuals with G/C and C/C genotype had higher BMI and diastolic blood pressure compared to those with G/G genotype.

**Conclusion:** Similarly to other reports the results of the present study revealed contradictory effects of the IL-6 174G/C polymorphism on different metabolic indices and could not provide a definite answer about its impact on obesity in Bulgarian sample and does not seem to be applicable as a marker of genetic predisposition to metabolic inflammatory diseases.

**SW01.W4–8**

**Inflammatory effects of resistin on human smooth muscle cells: up-regulation of fractalkine/CX3CR1 expression by TLR4 and Gi proteins pathways**

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**Aim:** Resistin is an adipokine with potent proinflammatory properties, acting as a potential regulator of inflammation. Several studies proposed resistin as a important link between obesity and insulin resistance. The obesity-related inflammatory state is linked to the increased risk of developing cardiovascular diseases and type 2 diabetes. The aim of this study is to determine whether resistin induces the expression of fractalkine (CX3CL1) and CX3CR1 in human smooth muscle cells (SMC) and to investigate the potential signaling pathways involved.

**Methods and Results:** Cultured human aortic SMC were stimulated with 100 ng/ml resistin for 4, 6, 12 and 24 h and then the CX3CL1 and CX3CR1 expression was assessed by quantitative RT-PCR and Western-blot. We found that resistin up-regulated CX3CL1 and CX3CR1 and induced the phosphorylation of p38MAPK and STAT3. Inhibitors of p38MAPK, JAK-STAT and NF-kB and AP-1 reduced significantly the CX3CL1 and CX3CR1 expression. Knockdown of STAT1 and STAT3 with decoy siRNA down-regulated the CX3CL1 and CX3CR1 expression. Anti-TLR4 antibody and pertussis toxin reduced CX3CL1 and CX3CR1 protein expression. The xCELLigence experiments revealed that resistin may use Gi-proteins for its effect on SMC. The resistin-induced CX3CL1 had chemotactic effect on monocytes transmigration.

**Conclusions:** Resistin contributes to the pro-inflammatory state of SMC by up-regulating CX3CL1 and CX3CR1 expression via a mechanism involving NF-kB, AP-1 and STAT1/3 transcription factors. Resistin employs TLR 4 and Gi proteins signalling for its effect on SMC. Resistin-induce CX3CL1 is functional in monocytes chemotaxis. The data reveal new mechanisms by which resistin contributes to the development of atherosclerosis.

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**SW01.W4–9**

Expression of genes encoding for sterol catabolism in *Mycobacterium* sp. VKM Ac-1817D producing 9-alpha-hydroxy androstenedione

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**Strain of *Mycobacterium* sp. VKM Ac-1817D is capable of oxidizing sterols (cholesterol, phytosterols) to form high-value 9α-hydroxy-4-androsten-3,17-dione (9-OH-AD) as a major product.**

In order to elucidate the genes involving in sterol catabolic pathways in this strain, a whole genome and whole transcriptome sequencing was carried out on Genome Analyzer IIX (Illumina) and HiSeq 2000. Automatical annotation of long contigs showed about 6100 ORF. Using reference genes of steroid catabolism of
relative actinobacteria, *Mycobacterium* and *Rhodococcus*, we found genes encoding the enzymes of steroid catabolism: enzymes accounting for the elimination of aliphatic side chain at C17, as well as for steroid core degradation, for sterol uptake and transcriptional regulators (kstR and kstR2).

The genes encoding for the key enzymes catalyzing steroid core oxidation in mycobacteria were analyzed in more detail, e.g. the two-subunit terminal oxygenase 3-ketosteroid-9α-hydroxylase (*KSH*) composed of a Rieske-domain containing oxygenase (*KSHA*) and a class IA ferredoxin reductase (*KSHB*), and 3-ke- 

steroid-Δ9-dehydrogenase (Δ9-KSTD). Five candidate genes of KSHA, two candidate genes of KSHB and four candidate genes of Δ9-KSTD were revealed.

The transcriptome analysis of the strain grown on glycerol in the presence of cholesterol versus the strain grown on glycerol solely showed that the expression of two *kshA* genes, one *kshB* and one Δ1-*kstD* rose in 8–90 times while the expression of other candidate ORFs remained as low as in the strain grown on glycerol solely.

In *silico* search of operons and analysis of promoters showed that only genes with high expression on cholesterol have binding sites of transcription repressors kstR and kstR2. Another candidate ORF has no such sites in the upstream regions. Therefore, the genes with maximal identity with reference genes kshA, kshB and *kstD* are the only ones which really involved in sterol catabolism in *Mycobacterium* sp. VKM Ac-1817D.

The results would contribute to better understanding of natural sterol catabolism by mycobacteria, but also to the genetic engineering of the strains producing key steroid intermediates for the pharmaceutical industry.

**SW01.W4–10**

*Sfp1* prion conversion is not equal to the absence of this yeast protein

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Yeast prions possess some features similar to those of the mammalian prion, PrP. But yeast prions raise different phenotypes, which are not necessarily associated with functional defects, and may even be detrimental to the cell under specific conditions. Interestingly, many of yeast prion proteins play roles in transcription or translation, and may execute an additional regulatory level in the cell.

[*ISP*] prion is formed by the Sfp1 protein, a global transcriptional regulator. This prion diminishes nonsense suppression caused by mutations in *SUP35* (yeast eRF3 gene). Apart from other yeast prions, Sfp1p aggregates are primarily localized in the nucleus, and [*ISP*] propagation does not depend on Hsp104.

It is believed that prion switch of the protein should cause the loss of its function. Nevertheless, the consequences of SFP1 deletion and Sfp1p prionization are dramatically different. Whereas sfp1Δ cells exhibit nonsense suppression, are more sensitive to translation inhibitors and grow much more slowly than [isp] cells, the [*ISP*] cells have clear non-suppressor phenotype, are more resistant to translation inhibitors and grow faster not only than sfp1Δ cells, but than [isp] cells as well. We found that these effects of Sfp1p prionization may be explained by enhanced *SUP35* expression in [*ISP*] cells. To further examine effects of Sfp1p prion conversion and establish the link between *SFP1* and *SUP35*, we conducted a whole-genome transcription profiling of [*ISP*], [isp] and sfp1Δ strains. We found that *SFP1* deletion caused differential expression of about 2000 genes in our strains, whereas about 300 genes were differentially expressed between [ISP] and [isp] strains (absolute logarithm fold change threshold 0.5, p < 0.001). The latter group includes among others the genes coding for cell membrane ion transporters and genes that are indirectly involved in translation. This data may help establish the link between the transcription factor Sfp1 and translation and understand whether this prion serves as a transient regulator of gene expression.

**SW01.W4–11**

Association of caspase-9 promoter region polymorphisms and breast cancer


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Breast cancer is most common malignancy in women. Caspase-9 (cas-9) plays a central role in intrinsic pathway of apoptosis. It is known that low expression or dysregulation of caspases might influence the apoptotic process and result in inappropriate cell proliferation. Single nucleotide polymorphisms are the genetic variations that may contribute to susceptibility to cancer. These variations may cause alterations on genes expression levels and functions. The aim was to determine if there is an association between cas-9 gene promoter region −1263A>G and −712C>T polymorphisms and breast cancer in Turkish patients. In this case-control study 80 breast cancer patients and age matched 55 healthy controls were included. The caspase-9 −1263A>G and −712C>T genotypes were determined using PCR and RFLP assays. There was no significant difference in the distributions of genotypes between cases and controls for the −1263A>G polymorphism. The C allele of the −712C>T polymorphism was associated with a significantly increased (p < 0.0001) risk of breast cancer. Compare to the caspase-9 polymorphisms and histopathologic characteristics (tumor type, invasion, lymph node involvement, histologic grade estrogen receptor, progesterone receptor and in situ component) of the patients, the AG genotype of −1263A>G polymorphism was associated with invasive ductal tumor type. In −712C>T polymorphism CC genotype was associated with progesterone receptors and lymph node involvement. Promoter region changes may affect expression of cas-9 gene. The changes of the cas-9 expression activity may cause the decrease of the apoptotic activity and the developing of the cancer. The results of this study suggest that casp-9 promoter region −1263A>G and −712C>T polymorphisms may affect the cas-9 expression and might be useful markers for determining genetic susceptibility to breast cancer.

**SW01.W4–12**

Effect of 5-HT on phenotypic transition of renal proximal tubular epithelial cells

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Platelet activation is observed in tissue damage occurs during various acute and chronic renal diseases. Recent studies suggest that some factors such as released from activated platelets may participate in inflammation and fibrosis observed after renal injury and renal proximal tubular epithelial cells give response to renal damage by changing their phenotypes. Although it has been known that serotonin (5-HT) is stored in platelets and released in the early phase of platelet activation, the effect of 5-HT release on proximal tubular epithelial cells have not been investigated yet.
In the present study, it was investigated whether platelets and platelet-released 5-HT are directly involved in the functional regulation of renal proximal tubular epithelial cells. Renal proximal tubular epithelial cells were prepared, characterized and treated with platelet lysate or 5-HT. The phenotypic transition of these cells was investigated. Since myofibroblasts express cytoplasmic alpha-smooth muscle actin (α-SMA) which is an actin isoform that predominates within vascular smooth-muscle cells and plays an important role in fibrogenesis, α-SMA protein was detected as a marker of proximal tubular epithelial cell differentiation into myofibroblasts. It was found that treatment of renal proximal tubular epithelial cells with platelet lysate or 5-HT increased the expression of α-SMA protein.

**SW01.W4–13**

**Association of hsp70-2 (+1267A/G) and hsp70-hom (+2437T/C) polymorphisms with cerebral atherosclerosis in Croatian population**

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The aim of this study was to test for a possible association of hsp70-2 (+1267A/G) and hsp70-hom (+2437T/C) polymorphisms with stenosis of cerebral arteries (SCA) in the Croatian population.

The genotyping of DNA, isolated from the whole blood of 51 patients with SCA and 48 healthy controls, was performed. Fragment size analysis upon restriction enzyme digestion was used for genotype/allele definition. The significance of findings was tested using the χ2 test.

hsp70-2 (+1267A/G) polymorphism was associated significantly with SCA. The results of genotyping analysis indicated that the genotype AG was associated preferentially with SCA (p = 0.033). No association for hsp70-hom (+2437T/C) polymorphism was found.

The study results support the existence of an association of hsp70-2 (+1267A/G) polymorphism with cerebral atherosclerosis. A higher frequency of the AG genotype in Croatian patients was observed. There was no evidence for an association of hsp70-hom (+2437T/C) polymorphism with cerebral atherosclerosis.

**SW01.W4–14**

**Effects of physical activity on DNA stability and production of reactive oxygen species**

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**Introduction:** Etiology of certain pathological processes may be related to decreased DNA stability and increased production of reactive oxygen species (ROS). Some data have shown that exercise decreases incidence of some oxidative stress related diseases. However, depending on exhaustion and frequency, effects of physical activity can differ.

**Aim:** Our objective was to determine is single soccer training able to disrupt balance between oxidants and antioxidants and thereby introduce the participants of the study in the state of oxidative stress causing DNA instability. We have also observed tested subjects after 45 days training period in order to investigate whether adaptive response will be developed and to what extent.

**Materials and Methods:** Sixteen soccer players (mean ± SD age: 18.13 ± 0.35 years) were included in this study. DNA comet assay was performed to observe possible change in DNA stability. Oxidative status of our subjects was estimated through blood levels of superoxide anion, the thiobarbituric acid-reacting substances (TBARs), total antioxidant status (TAS), total oxidant status (TOS), prooxidant–antioxidant balance (PAB) and sulfhydryl-groups (SH-groups).

**Results:** DNA score and percent of cells with medium and high damage were increased after training but without significance. On the contrary, SH –groups, TOS and TC levels were lower after training, and these changes did reach statistical significance (p = 0.033, p < 0.001 and p = 0.002 respectively). PAB was also decreased after training (p < 0.045). Percent of cells with medium and high DNA damage was significantly lower after 45 days training period (p = 0.01). TOS and MDA levels have changed over time toward lower values with significant differences (p < 0.001, p = 0.038, respectively). SH–groups levels have also been changed significantly, but toward higher values (p = 0.006).

**Conclusion:** This study demonstrated that, because of daily exposure to physical activities, single training session hadn’t led to DNA instability, and possible oxidative stress development was quickly neutralized by antioxidative mechanisms. We have also supplied data that during 45 days training period adaptive response was induced.

**SW01.W4–15**

**The draft genome sequence of Bacillus cereus F strain, isolated from ancient permafrost sample**

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Permafrost covers nearly 50% of Russia’s territory. It can reach ages of several million years harboring unique microbiota. Still little is known what potentials for biotechnology and possible threats to environment these microorganisms can present.

Mammoth Mountain is situated on Aldan River in Yakutia and represents an unique geological object, where horizons of intact permafrost aged from 40 000 to 10 million years are exposed annually due to river bank landfill. A viable strain ‘F’ of Bacillus cereus sp. was isolated from a sample of permafrost aged from 3 to 5 million years. Though Bacillus cereus is capable of forming spores it is still seems astonishing how a bacteria can survive natural soil radiation for some million years under temperature nearly –3°C and in a condition of almost total deprivation of energy sources.

Here we report the first draft sequence of Bacillus cereus F genome. The sequencing was performed with use of hybrid approach: a rapid fragment library was sequenced on a Roche FLX instrument followed by sequencing of 2 × 50 bp mate-paired library on SOLiD v. 3.5 instrument. This allowed us to perform scaffolding and error correction in Roche FLX reads, occurred especially in homopolymer stretches, resulting in 5.26 Mbase genome, presented by 187 contigs.

Nucleotide sequences: This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AHHI00000000. The version described in this paper is the first version, AHHI01000000.
SW01.W5 Nucleic Acid Targets and Therapeutics (I-W5)

SW01.W5–1 Oligonucleotide synthesis interfaced with molecular biology
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Chemically modified, synthetic oligonucleotides (ODNs) act in a sequence-specific manner to modulate gene expression. Here we describe new analogs with unique biological and chemical properties and also new methods for the synthesis of DNA and RNA up to 300 nucleotides in length.

3′-O-(N,N-diisopropylamino)ethynylphosphonates were used to synthesize ethynyl phosphites. Post synthesis, triazole was introduced with an azide and CLICK chemistry (1, R1 = H,lysyl,peptidyl,CH2CH2N(CH3)3). Of particular interest was that a 16 mer with four triazoylphosphonate linkages was transfected without lipid into HeLa, WM-239, SK-N-Fi, and Jurkat cells. These ODNs were active as microRNA antagonomers.

Synthesis of boranephosphonamide ODNs begins with 3′-O-bis (dialkyamino)phosphino-2′-deoxynucleosides. Condensation generates a PH1 dimer which is boronated. Extension to ODNs can then be carried out. These analogs are nuclease resistant, form stable duplexes with DNA, and are biologically active.

Boranephosphonate DNA has been synthesized using a new, unpublished procedure. This method allows synthesis with standard phosphoramidites on supports, including acidic removal of trityl groups. This analog is biologically active in RNA interference and as microRNA antagonomers.

Boran containing ODNs reduce Au3+, PdCl2, and Ag+. The products of these reductions are the metals, boronic acid, and intact, natural internucleotide linkages (and phosphate triesters if reduction is in alcohols). Boranephosphonate DNA can be incorporated into arrays having double crossover junctions and reduced to generate silver nanoassemblies useful in diagnostics and nanotechnology.

In collaboration with Emily LeProust and Doug Dellinger at Agilent Technologies, we have developed procedures for the synthesis on glass slides of DNA & RNA 300 nucleotides in length (approximately 50% fidelity). Phosphoramidites are deposited by a modified ink jet printer and synthesis finished in a flow cell. Approximately 6 billion couplings on Agilent instruments are completed each 24 h.

SW01.W5–2 Anticancer siRNAs
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A substantial progress in the study of cancer development was achieved during last decades. It is likely that chemotherapy today reaches the limit of its efficiency; hence the necessity of the time is development of the new approaches for cancer cure. Therapeutic nucleic acids, namely gene-targeted and antisense oligonucleotides, small interfering RNAs (siRNAs), ribozymes, DNAzymes, etc., targeted cancer associated or cancer inducing genes can be considered as innovative approaches for therapy of cancer. Application of siRNAs targeted to the genes coding for key cell cycle regulators revealed that suppression of these genes have different influence on the proliferation of different human cancer cells. We show that silencing of HER2, PKC, and Cyclin B1 substantially reduces the growth rates of all cell lines under study except for HL-60 cells but does not induce cell death or apoptosis. anti-Cyclin B1 siRNA induces the most pronounced inhibition of cell division in neuroblastoma SK-N-MC cells; moreover the substantial retardation of cellular division maintain even after restoration of the initial level of this gene expression. Although HER2 is the recognized target for breast cancer gene therapy, results obtained in RNAi experiments indicate that PKC is more advantageous target: a two-fold decrease in its expression suppresses cell proliferation rate of MCF-7 cells by an order of magnitude. Anti-MDR1-siRNAs were found to inhibit expression of p-glycoprotein and to reverse multiple drug resistance phenotype of cancer cells. The developed anti-MDR1siRNA induce death of cancer cells in the presence of 30-times lower doses of cytostatics than the doses well tolerated before. The approached to overcome challenges associated with nuclease stability, selecting effective sequences and achieving efficient delivery to target cells and tissues will be discussed.

This work was supported by RAS programs 'Molecular and Cellular Biology' and 'Basic sciences for medicine', grant from SB RAS No. 41, RFBR Nos. 08-04-01073-a, 08-04-00753-a, 09-04-12128-ofi_m; Scientific Schools grant No. 2972.2012.4.

SW01.W5–3 Multifunctional interleukin-6 receptor specific DNA and RNA aptamers
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Aptamers are nucleic acids with the capacity to interact with a huge diversity of all possible conceivable targets. Usually those nucleic acids cannot pass bio membranes and thus enter cells. Provided aptamers enter specific cells by targeting receptors, which usually are internalized. Afterwards the formed aptamer-receptor complex might be co-internalized due to cellular processes. This could be used for cargo delivery of molecules of interest via linkage to the aptamer.

We have selected an interleukin-6 receptor (IL-6R) specific RNA aptamer consisting of 19 nucleotides, which may serve as cargo vehicle [1]. Additionally, we succeeded in selecting an IL-6R specific DNA aptamer exhibiting the 16 nucleotide long sequence d(GGGT)4[2].

Both, the DNA and RNA aptamer seem to comprise structure similarities as they are forming parallel G-quartets, which could be shown by CD spectroscopy and melting experiments, but, in contrast to the RNA aptamer, the DNA aptamer is not internalized.

Moreover, d(GGGT)4 was already known as HIV inhibitor previously. Confirming this, we could show an efficient inhibitory effect on HIV de novo infection for the RNA aptamer as well.

References
**SW01.W5–4**
The structural basis for the induction of nucleotide flipping-out, a sharp bend and a left-handed twist in CGG triplet repeats by actinomycin D binding
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The potent anticancer drug actinomycin D (ActD) functions by intercalating into DNA at GpC sites, thereby interrupting essential biological processes including replication and transcription. Certain neurological diseases are correlated with the expansion of (CGG)\textsubscript{n} trinucleotide sequences, which contain many contiguous GpC sites separated by a single G:G mispair for ActD binding. To characterize the binding of ActD to CGG triplet repeat sequences, the structural basis for the strong binding of ActD to neighbouring GpC sites flanking a G:G mismatch has been determined based on the crystal structure of ActD bound to ATGCGGCAT, which contains a CGG triplet sequence. The binding of ActD molecules to GCGGC causes many unexpected conformational changes including nucleotide flipping out, a sharp bend and a left-handed twist in the DNA helix via a two site-binding model. Heat denaturation, circular dichroism and surface plasmon resonance analyses showed that adjacent GpC sequences flanking a G:G mismatch are preferred ActD-binding sites. In addition, ActD was shown to bind the hairpin conformation of (CGG)\textsubscript{16} in a pairwise combination of ActD-binding sites. In addition, ActD was shown to bind with greater stability than that of other DNA intercalators.

**SW01.W5–5**
New potentially targets to for inhibition of metastatic and invasion capacity of gastric cancer cells
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**Background:** The aim of the study was to investigate the biological significance of S100A2, KRT17 and SALL4 genes in gastric cancer pathogenesis. All three genes were selected based on their high expression level on gastric adenocarcinoma samples.

**Methods:** The role of S100A2, KRT17 and SALL4 genes was investigated after knocking down their expression, using RNA interference technology, in two human gastric cancer cell lines (AGS and NCI-N87). The inhibition of specific mRNA expression was determined by quantitative PCR and the effect of gene down-regulation on signaling pathways was assessed by western-blotting. Cell proliferation (CellTiter 96 AQueous One Solution Cell Proliferation Assay), apoptosis (anexin V/PI assay) and invasion (matrigel invasion and scratch assays) were assessed to reveal the biological significance of targeted genes in gastric cancer.

**Results:** The results showed that siRNA knockdown decreased the invasion and migratory capacity of tumoral cells depending on the cell line, without any significant effect on cell viability or proliferation. S100A2 inhibition decreased the invasion capacity of AGS cells by 19.31 ± 6.7%, and in combination with KRT17 knockdown reduced the cellular motility by 33.92 ± 6.7%. Simultaneous inhibition of all three target genes reduced the invasion capacity by 32.33 ± 5.1%. Results of western-blotting showed an increased phosphorylation level of β-catenin (S33/S37) and p-ERK1/2 ([T202/Y204]/[T185/Y187]) when inhibiting all three target genes. The increase of β-catenin phosphorylation level suggests that β-catenin was targeted for degradation via ubiquitin proteasome pathway, and therefore Wnt/β-catenin pathway, known to be involved in cell migration and invasion, was also inhibited. Activation of ERK1/2 may explain the lack of inhibitory effect on proliferation. This might occur due to existent feedback mechanism between Ras/MEK/ERK pathway and AKT/mTOR, known to be activated by KRT17.

**Conclusions:** S100A2, KRT17 and SALL4 genes appear to primarily mediate cell invasion and motility without any influence on cell viability. Collectively, these data suggest that selected genes may be potential targets for reducing metastatic capacity of gastric cancer cells.

**SW01.W5–6**
The role of transcription factor ATF3 in cardiac hypertrophy
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ATF3 is a stress induced transcription factor from the basic leucine zipper (bZIP) family. ATF3 is an immediate early gene that responds to extracellular signals. ATF3 mRNA level is relatively low in most cell types but greatly increases upon induction by a variety of extracellular signals such as serum, EGF, TPA, cytokines and multiple stress stimuli.

ATF3 transgenic mice display cardiac hypertrophy, fibrosis and arrhythmia. These ATF3 transgenic mice expressed ATF3 under the control of the α-myosin heavy chain (α-MHC) promoter. The αMHC promoter becomes active at embryonic day 10 in the atria and striated muscle surrounding the heart, and in the ventricles only 12 h before birth. This expression pattern did not allow the distinction between developmental effect and postnatal expression of ATF3 on the pathogenesis of cardiac hypertrophy.

Cardiac hypertrophy is usually considered a poor prognostic sign and is associated with nearly all forms of heart failure. In response to changes in functional load the heart triggers a hypertrophic response to counterbalance the increase in wall stress.

The exact molecular mechanisms responsible for the enlargement of the heart are not clear, but understanding them is important for the development of novel treatments.

Recent findings from our lab show that ATF3 is found at the receiving end of multiple neurohumoral heart stimuli. In addition, ATF3 KO mice display a lower degree of cardiac hypertrophy following chronic exposure to pressure overload.

We have generated ATF3 transgenic mice using the tetracycline binary system that enables regulated cardiac expression of ATF3. Generating transgenic mice with cardiac regulated ATF3 expression will allow us to examine the effect of transient expression of ATF3 on heart hypertrophy. In addition, it will enable us to reveal the downstream targets of ATF3 leading to cardiac hypertrophy and identification of ATF3 target genes. ATF3 is a stress induced transcription factor from the basic leucine zipper (bZIP) family.

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**SW01.W5–7**

*Cas9 – a programmable RNA-guided DNA endonuclease from the bacterial adaptive immune system*

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Clustered regularly interspaced short palindromic repeats (CRISPR) together with CRISPR associated (cas) genes form an adaptive prokaryotic immune system which provides acquired resistance against viruses and plasmids. CRISPR consists of arrays of short conserved repeat sequences interspaced by unique DNA sequences called spacers. The CRISPR-Cas system functions by acquiring short pieces of foreign DNA as new spacers and subsequently uses them as templates to generate specific small RNA molecules (crRNA) which combined with Cas proteins into effector complexes that trigger degradation of foreign nucleic acid. In Type I and Type III CRISPR systems, nucleoprotein complexes involved in crRNA-mediated silencing of foreign nucleic acids are comprised of large multisubunit aggregates. We show that in Type II systems, the silencing complex consists of a single Cas9 protein [1], which binds to crRNA/tracrRNA to mediate sequence-specific cleavage of invasive dsDNA.

We isolated the Cas9-crRNA complex of the *S. thermophilus* CRISPR-Cas system and demonstrated that it generates *in vitro* a double strand break at specific sites in target DNA molecules that are complementary to crRNA sequences and bear a DNA motif. We show that DNA cleavage is executed by two distinct active sites (RuvC and HNH) within Cas9, to generate site-specific nicks on opposite DNA strands. Sequence specificity of the Cas9-crRNA complex is dictated by the 42 nt crRNA. All together our data demonstrate that the Cas9-crRNA complex functions as an RNA-guided DNA endonuclease [2]. The simple modular organization of the Cas9-crRNA complex, where specificity for DNA targets is dictated by a small crRNA and the cleavage machinery consists of a single, multidomain Cas protein, provides a versatile platform for the engineering of programmable RNA-guided DNA endonucleases. These findings pave the way for the development of novel molecular tools for RNA-directed DNA surgery [3].

**References**


**SW01.W5–8**

*Ku protein as an intracellular target of extracellular DNA*

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Biological effects of extracellular DNA (exDNA) suggest its interaction with the effector molecules exposed on the cell surface or localized inside the cell. We have earlier demonstrated that substantial amount of exDNA is bound to the cell surface (csbDNA) via DNA–protein interactions and this DNA inhibits polyIC-induced production of proinflammatory cytokines in the human umbilical vein endothelial cells (HUVEC) [1]. The goal of this work was to identify the cellular targets of immunoinhibiting csbDNA using ODNs containing the nucleotide motifs frequently found in csbDNA and displaying the same effects.

The binding of [32P]-labeled single- and double-stranded ODNs (ss- and ds-ODNs) with membrane-cytosolic extracts and living human umbilical vein endothelial cells (HUVEC) was studied by electromobility shift assay (EMSA). Complexes of biotinylated ODNs with target proteins were affinity isolated using streptavidin sepharose with subsequent SDS-PAGE and identified by MALDI-TOF mass spectrometry.

Both ss- and ds-ODNs form strong ODN–protein complexes with similar electrophoretic mobilities after incubation with the membrane-cytosolic extracts of HUVEC either when added extracellularly or lipofected into cells. ODN-binding proteins were identified as DNA-binding components of DNA-dependent protein kinase (DNA-PK), namely, Ku70 and Ku80 proteins. Diverse cellular localizations and functions of Ku protein demand further clarification of Ku70/80 role as a mediator of the csbDNA immunoinhibiting effects.

**Reference**


**SW01.W5–9**

*Gene Mbl1 is a target of interferon alpha*

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**Background:** Interferon alpha (IFNa) is a cytokine with a wide range of activities, but not all of its target genes are still identified. The IFNa target genes are regulated by dominating transcription factor ISGF-3. The genome wide search in rat genome for genes containing ISGF-3 binding sites in their promoters was conducted by conservation-aided transcription factor binding site finder, http://biomed.org.ua/COTRASIF/ and a list of 162 genes was obtained.
The aim of our study was to find out experimentally not confirmed IFNα target genes from this list and to verify the most reliable candidates. Methods: Gene Ontology (GO) enrichment analysis was performed with FatiGO. The experimental assay of candidate gene and IFNα gene expression were conducted by qRT-PCR in real time. The total RNA was isolated using Trizol reagent from primary hepatocytes cultivated with IFNα (250 U/ml) during 3 h and from liver of the rats treated with PolyI PolyC (25 μg/100 g of body weight) during 20 h. Results: The only enriched GO category among 162 genes was immune response (GO:0006955), containing nine genes. Only two of them (PF-4, Mbl1) were not experimentally confirmed as IFNα targets. Mbl1, our gene of current interest, encodes mannoselocking lectin initiating lectin pathway in complement system. We have shown that up-regulation of IFNα mRNA is accompanied by down-regulation of Mbl1 mRNA level in both cases particularly 2.3-fold increase of IFNα mRNA in in vitro IFNα-treated hepatocytes was accompanied by decrease of Mbl1 mRNA content for 35%, while corresponding values in the liver of PolyI-PolyC-treated rats are fivefold augmented IFNα mRNA versus decrease for 25% of Mbl1 mRNA content. Conclusion: We suggest that IFNα negatively regulates Mbl1 gene expression presumably via two ISGF-3 binding sites in the promoter of this gene. The more direct evidences of Mbl1 gene regulation by IFNα are under investigation. Reference 1. Tokovenko B. et al. (2009) Nucl. Acids Res, 37, N 7, P. 49.

SW01.W5–10
Expression and regulation of STEAP1 and STEAP1B in prostate cell lines through mRNA and protein stability and epigenetic mechanisms
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Background: STEAP1 is over-expressed in several types of tumors, particularly in prostate cancer, and it can be found in the plasma membrane of epithelial cells, at cell–cell junctions. Taking into account that STEAP1B expression in prostate cells and the mechanisms underlying STEAP1 regulation are yet to be ascertained, we aim to compare the expression between STEAP1 and STEAP1B in human prostate cell lines, as well as to analyze the stability of STEAP1 mRNA and protein between non-neoplastic and neoplastic prostate cells. In addition, we intend to explore the involvement of epigenetic mechanisms in the regulation of STEAP1.

Results: STEAP1B mRNA isoforms, STEAP1B1 and STEAP1B2, are differentially expressed on human prostate cell lines, with higher expression on LNCaP cells, followed by PNT1A, PC3 and PNT2. STEAP1 protein is also expressed in PNT1A cells, but has a lower molecular weight when compared to LNCaP cells. Regarding regulation of STEAP1, our results show that the stability of STEAP1 mRNA and protein is higher in LNCaP than in PNT1A cells. A comparative analysis of the methylation pattern of PNT1A and LNCaP cells CpG islands located on the promoter region of STEAP1 gene is underway.

Conclusion: Here we demonstrate for the first time that STEAP1B1 and STEAP1B2 isoforms are expressed in prostate cells, being also over-expressed in LNCaP cells. STEAP1 mRNA and protein stability could be an important control point for the regulation of STEAP1 gene expression.

SW01.W5–11
New fluorescent probes based on minor groove binders and cyanine fluorophores: synthesis and interaction with the target dsDNA
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New fluorescent probes based on hairpin and tandem polyamide minor groove binders were synthesized using solid-phase synthesis and click chemistry. For their fluorescent labeling, two commercial and four new synthetic polymethylene cyanine fluorophores (two intercalators and two minor groove binders) were used. Their interaction with a target fragment of murine pericentromeric satellite dsDNA was characterized in vitro by the native gel shift electrophoresis, thermal denaturation, circular dichroism and fluorescent spectroscopy methods. All the probes demonstrated quite good affinity for the target DNA (Kd being in micromolar range), stabilization of the target duplex in thermal denaturation experiments up to 24°C, hairpin conformation of the ligand in the complex with dsDNA and strong increase of the fluorescence intensity upon interaction with the target DNA. Effect FRET was observed when two adjacent probes labeled by Cy3 and Cy5, respectively, were in complex with a target. Fluorescent labeling by both intercalating and minor groove binding fluorophores affected the affinity of ligands in different degree. Taking into account the ability of polyamides to penetrate into cells, the synthesized ligands can be potentially applied for detection and visualization of repetitive genomic DNA regions (as centromers or telomers) on isolated chromatin, chromosomes or in live cells. However, the structure of the ligands and their sequence needs to be optimized in order to increase their affinity and specificity.

SW01.W5–12
The interaction of lipophilic derivatives of siRNA with hematopoietic and tumor cells
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siRNAs are considered to be promising therapeutic agents for sequence-specific silencing of disease-related genes. However, the problem of siRNAs delivery into cells limits their biomedical application. Conjugation of siRNA to the molecules, which can be internalized into the cell by natural transport mechanisms, can result in the enhancement of siRNA cellular uptake. In this work we investigated the carrier-free accumulation of nucleic acid-resistant siRNA equipped with lipophilic residues tethered on the 5′-end of the sense strand in KB-8-5 cancer cells and normal hematopoietic cells. We showed that conjugates of siRNA (0.2–5 mM) with cholesterol effectively penetrated (up to 100%) into the cells, whereas the uptake of the unmodified siRNA was insignificant. The efficiency of the carrier-free cellular accumulation of lipophilic siRNAs is dependent upon the type of lipophilic residues, the type of the target cells and the length of
Abstracts

SW01.W5–13
Inhibition of STAT3 expression via chemically modified siRNA’s enhances the effects of cisplatin in the parental and resistant Calu1 non-small cell lung cancer cells

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Background: Lung cancer is still the most common fatal cancer in the world and unfortunately many cases are diagnosed when the disease is already at a locally advanced or metastatic stage. Chemotherapy is the most important option in the treatment of lung cancer, but resistance to cisplatin is the major obstacle that limits its use. Activated STAT3 gene expression has been reported in several primary cancers and cell lines and targeting the STAT3-signaling pathway is a new therapeutic approach in the anticancer therapies. We aimed to investigate the effects of STAT3 silencing on cell proliferation and cisplatin resistance in the lung cancer cells.

Methods: Non-small cell lung cancer cell line Calu1 and its cisplatin resistant subline CR-Calu1 were used to reveal the effects of STAT3 inhibition with chemically modified anti-STAT3-siRNA’s. qRT-PCR (Quantitative real-time reverse transcriptase-polymerase chain reaction) and western blot assays used to assess STAT3 expression both at mRNA and protein levels. Apoptosis was evaluated by caspase-3 enzyme activity and cell death assays.

Results: Our results have shown that STAT3 expression significantly increases in the cisplatin resistant subline and silencing the STAT3 expression provides increased induction of apoptosis via caspase3 activation. Cisplatin sensitivity was increased significantly in Calu1 and CR-Calu1 cells and cisplatin IC50 values were decreased from 17.23 to 15.50 μM for Calu1 and STAT3-siRNA transfected Calu1; from 116.80 to 17.52 μM for CR-Calu1 and STAT3-siRNA transfected CR-Calu1.

Conclusions: Our results show that, when anti-STAT3 siRNAs and cisplatin are applied together, cisplatin response and apoptosis can be significantly increased in parental and cisplatin-resistant NSCLC cells. We therefore propose that the combined treatment of the conventional chemotherapeutic drug cisplatin with STAT3 inhibitors can help to overcome chemotherapeutic resistance in lung cancer.

Gold nanoparticles are effective tools to transfect cells for gene therapy. Here, gold particles are conjugated with transferrin in order to target estrogen receptor specific antisense oligonucleotides. Gold nanoparticles are activated by thiol groups which enabled the attachment of both a protein and an oligonucleotide. Gold particles in cells were visualised by microscopy. Cell proliferation was detected by MTT and estrogen receptor expression was measured by real-time PCR. Human breast cancer cells expressing estrogen receptor, MCF 7 have efficiently taken up these conjugates possibly though the uptake of transferrin receptors. The antisense oligonucleotides against estrogen receptor were able to suppress cell proliferation as well as estrogen receptor expression. Further studies are being carried out using this delivery system against other overexpressed breast cancer specific genes and their messenger RNA.

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SW01.W5–15
The suppressor of cytokine signalling-2 regulates the effects of estradiol on body growth

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The Suppressor of Cytokine Signaling (SOCS)-2 protein acts as a negative regulator of the effects of GH-STAT5b signalling on body growth in vivo [1]. SOCS2 null mice (SOCS2KO) are characterized by a 30–50% increase in body weight in the absence of increased circulating levels of GH or IGF-1. Importantly, whereas SOCS2KO mice have enhanced growth, combined STAT5bKO and SOCS2KO mice do not, a demonstration that STAT5b is needed for the excess of body growth observed in SOCS2KO mice. Estrogens and GH interact closely to regulate pubertal growth [2]. 17β-Estradiol (E2) modulates GH actions in liver by acting centrally, regulating pituitary GH secretion, and, peripherally, modulating GH signalling. E2 induces SOCS2 expression which is followed by inhibition of GH-STAT5 signalling [3]. This mechanism suggests that SOCS2 could play a role in regulating the inhibitory effects of estrogens on GH-regulated somatic growth. To test this hypothesis, we evaluated the effects of E2 on body weight and growth in SOCS2KO and C57BL/6 (WT) mice after E2 benzoate administration (50 mg kg⁻¹ 48 h; sb) during 15 weeks. In WT mice, E2 increased the hepatic level of SOCS2 mRNA up threefold whereas it was undetectable in SOCS2KO mice. In contrast, C5, SOCS1, and SOCS3 genes were expressed in both phenotypes. E2 increased SOCS1 mRNA level (fourfold) in WT but decreased it in SOCS2KO mice. E2 increased the hepatic mRNA levels of female-specific genes (i.e., A1bg, Cyp2b9) and IGF-1 in WT and SOCS2KO mice. However, E2 prevented the gain of body weight and growth in WT but not
in SOCS2KO mice, which suggests that SOCS2 regulates the inhibitory effects of E2 on body growth in vivo.

References

SW01.W5–16
Cationic liposomes for efficient delivery of nucleic acids
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Gene therapy as an alternative to the conventional medicine implies the elimination of the cause of a disease via the introduction of DNA or oligonucleotides (antisense oligonucleotide or siRNA) into a target tissue. Cationic liposomes are attractive systems for gene delivery. However, their efficiency in a biological environment is often limited or reduced by the interaction with several extra- and intracellular barriers. In extracellular space the nucleic acid/lipid complex (lipoplex) is met by blood components and proteins of the immune system, which can destabilize the lipoplex and induce the untimely release of DNA. Endosomal and nuclear membranes can be referred to as the main intracellular barriers for liposomal nucleic acids delivery.

The ability of natural polyanamines (spermidine, spermine) to bind DNA and to pack it into small dense particles was used in order to create transporting agents for the delivery of nucleic acids into cells. The presence of four amino groups with different pKa values in spermine-based amphiphiles protect the nucleic acid from degradation and facilitate its escape from the endosome. We have elaborated a convenient method for synthesis of spermine-based cationic amphiphiles.

To assess the potential of the synthesized spermine-based amphiphiles as new gene delivery systems, we estimated the biological effects of these compounds on cell viability and cytosolic delivery of nucleic acids. The cationic liposomes composed of the cationic amphiphiles and DOPE provided delivery of fluorescein isothiocyanate-labeled oligonucleotide, plasmid DNA and siRNA into HEK293 cells with an efficiency significantly higher than that of Lipofectamine 2000. The amphiphile containing two cholesterol units, carbamate linker and spacers demonstrated the best in vitro transfection activity among other analogues tested and was defined as a promising candidate for further transfection studies to be hold in vivo.

SW01.W5–17
Exosomes are natural carriers of exogenous siRNA to human cells in vitro
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Exosomes are nano-sized vesicles of endocytic origin that are involved in cell-to-cell communication including shuttle RNA, mainly mRNA and microRNA. As exosomes naturally carry RNA between cells, these particles might be useful in gene cancer therapy to deliver therapeutic short interfering RNA (siRNA) to the target cells. Despite the promise of RNA interference (RNAi) for use in therapy, several technical obstacles must be overcome. The major hurdle of RNAi-based therapeutics is to deliver safe nucleic acids across the cell plasma membrane. Exosomes can potentially be used as a safe, efficient, organ/cell-specific and nonimmunogenic siRNA delivery vector.

This study demonstrates that exosome vesicles derived from human can deliver siRNA to recipient cells in vitro. Having tested different strategies, an optimized method was used to introduce siRNAs into human exosomes of various origins. Exosomes from culture medium and malignant effusions were used to transport siRNA to human cells. The delivery of siRNA via exosomes to cells was confirmed using confocal microscopy and flow cytometry. The fluorescently labeled siRNA was detected in recipient cells following incubation with exosomes. The exosome-delivered siRNA was mainly localized in the cytoplasm of the recipient cells. Two different siRNAs against RAD51 and RAD52 were used to transfect into the exosomes for therapeutic delivery into target cells. To examine whether the exosome-delivered siRNA was effective at causing post-transcriptional gene silencing in recipient cells, the gene products of RAD51 and RAD52 were analyzed by immunoblotting. Moreover, the exosome-delivered siRNA against RAD51 was functional and caused the massive reproductive cell death of recipient cells.

In summary, the results strongly suggest that exosomes effectively delivered the siRNA into the target cells, causing selective genes’ silencing and leading to reproductive cancer cell death by knockdown of RAD51. Therefore, the results of this study can be seen as a proof of the ability to use the human exosomes as vectors in gene therapy, especially given that exosomes derived from human are nearly no immunogenic, which makes them an ideal candidate for gene therapy, including RNAi-based cancer therapeutics.

SW01.W5–18
A component of transcriptional PRC2 complex, enhancer of zest homology (EZH2), modulates endothelial cell responses to hypoxia and post-ischaemic angiogenesis in a mouse model of limb ischaemia
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Endothelial cells (ECs) have major role in post-ischaemic angiogenesis. Trimethylation of lysine 27 of histone3 (H3K27me3) is a repressive epigenetic mark carried by EZH2 enzyme, the catalytic part of Polycomb complex 2 (PRC2, also comprising the suppressor of zeste 12 homolog-Suz12). We investigated how do in vitro hypoxia and in vivo limb ischaemia affect PRC2 expression in ECs and in the mouse limb muscles, respectively. The modulation of angiogenic genes by EZH2 and the angiogenic responses were assessed using EZH2 inhibitor, 3-deazaneplanocin (DZNep). Human umbilical vein ECs (HUVECs) were cultured under normoxia or hypoxia (1.2% O2, 6–48 h), to mimic ischaemia. HUVECs were treated with DZNep (control: 1% DMSO) or transfected with siRNA against EZH2 (control: scramble oligos) and migration was assessed by scratch assay. PRC2 protein levels were determined. The expression of PRC2 and proangiogenic/endothelial genes, eNOS, BDNF, VEGF and VEGF-receptor2, was measured by qPCR. Chromatin-immunoprecipitation cou-
pled with qPCR was performed for EZH2, Suz12 or H3K27me3 at promoters of aforementioned genes. Further, limb ischemia (LI) was used as a mouse model of in vivo angiogenesis. CD1 male mice (aged 15 week) received DZNep (1.5 mg/kg, i.p. every 2 days) or vehicle 1 day pre-LI. Post-ischemic blood flow (BF) recovery was assessed by colour laser Doppler at 30 min and weekly thereafter for 3 weeks. Mice were culled and tissue was snap-frozen or formalin-fixed for analyses.

Hypoxia increased EZH2 and H3K27me3 levels in HUVECs. DZNep and EZH2 knock-down reduced expression of EZH2 but not Suz12. These same treatments reduced the enrichment of EZH2, Suz12 or H3K27me3 at EC promoters, while improving HUVEC migration. LI has increased expression of EZH2 and H3K27me3 levels; which were reversed by DZNep. Post-ischaemic BF recovery (week 1–3) has significantly increased (p < 0.01) due to DZNep, which further increased the capillary density in the LI-muscles.

Therefore, inhibition of EZH2 promotes angiogenesis in both ECs and LI-muscles by enhancing the expression of pro-angiogenic genes.

**SW01.W5–19**

**Retinoid signaling is implicated in early hepatic regenerative response**

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Preliminary studies of liver regeneration induced by partial hepatectomy (PHE) identified a substantial depletion of hepatic retinoid stores, by >70%, in regenerating livers of wild type C57BL/6J mice. To understand this, we compared responses of wild type and lecithin:retinol acyltransferase (Lrat)-deficient mice, which totally lack hepatic retinoid stores, to PHE. Concentrations of all-trans-retinoic acid were significantly lower in the livers of Lrat−/− mice following PHE and this was accompanied by diminished expression of known retinoid-responsive genes compared to wild type mice. The Lrat-deficient livers showed delayed regeneration in the first 24 h after PHE and the regenerating liver weights of the Lrat−/− mice never exceeded 80% of the corresponding masses of wild type C57BL/6J livers. For C57BL/6J mice, the liver mass was fully restored within 72 h, whereas, for the Lrat−/− mice full liver mass regeneration was observed only at 7 days. The proliferative responses in Lrat−/− livers were impaired throughout this initial period of regeneration. At 12 h after PHE, we observed significantly less mRNA expression for growth factors and cytokines implicated in regulating the priming phase of liver regeneration, specifically for Hgf and Tgfs, but not Tgfβ. Compared to wild type mice, the temporal changes in mRNA levels for p21 and cyclins E1, B1 and A2 mRNAs and for hepatocellular BrdU incorporation and mitoses were delayed (i.e. shifted to later times) in Lrat−/− liver. To assess further the role of retinoid signaling in early hepatic regenerative response we used an approach involving expression of a dominant/negative retinoic acid receptor isoform (RARxDN). Mice with hepatocyte-specific ablation of Drosophila melanogaster (w) eyeless (Alb-Cre+/−) mice showed more than 50% lethality within 48 h after PHE and the rate of liver mass regain never exceeded 60% of pre-PHE values. Collectively, our data establish that hepatic retinoids and retinoid signaling are required for assuring an optimal early hepatic regenerative response by maintaining expression of important signaling molecules which regulate cell proliferation and differentiation immediately after hepatic injury.

**SW01.W5–20**

**Gene signatures in cancer may also overlap at the level of the product special domain organization and function**

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Studying of the chimera protein BCR-ABL as a result of translocation and subsequent fusion of genes belonging to different chromosomes and functioning as an oncogene causing chronic myeloid leukemia allowed to pinpoint different domains in the organization of this fused protein molecule. When investigating differently rearranged variants of this fused protein it turned out that the rearranging giving rise to the protein p210 unlike that of the protein p190 maintained special PH domain intact that was absent from the p190. Structural study of the PH domain discovered that α-helices is a predominant structure of this domain. This makes it looking like PDZ domain known to function as a molecular glue. Interestingly, when studying the intracellular localization of these products p210 was concentrated around the nucleus as if due to PH domain anchoring whereas p190 was distributed all over the cytoplasm. The first case was clinically beneficient unlike the second one. This observation poses a question on the possibility of some protein domains coded for by different genes to mimic some functional outcome of genetic activity depending on some personalized factors. Moreover, the specific localization of p210 poses a question on nucleus supplement with DNA synthesis precursors and the ability of ubiquitin special protease USP1 to recognize in the p210 anchorage the target of its activity that is PH domain of BCR-ABL fusion protein.

**SW01.W5–21**

**Inhibition of HIV-1 reverse transcriptase and its binding to HIV-1 integrase by modified oligonucleotides**

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During the early stages of HIV-1 replication two viral enzymes are activated: reverse transcriptase (RT) and integrase (IN). RT catalyzes the synthesis of DNA copy of the viral genomic RNA, and IN stimulates the viral DNA integration into the cell genome. Inhibition of the activity of these proteins should completely block viral replication right after the infection. Earlier we have found that short single-stranded 11-mer 2′-O-methylated oligonucleotides (mODNs) conjugated to 2,4,5,7-tetramethylfluorescein (eosin) inhibited the IN activity in vitro in nanomolar concentrations. Moreover the complexes of these compounds with a cell-penetrating peptide were shown to penetrate into HIV-1-infected cells, to block the viral replication at the reverse transcription step, and to lead to the emergence of drug resistance mutations known to confer a high level of virus resistance to non-nucleoside RT inhibitors.

In the present work we study interactions of mODNs with RT in vitro. Their impact on the RT catalytic activity was evaluated. It was clarified that the mODNs conjugation with eosin significantly increased their inhibitory potency. The conjugates were able to inhibit the activity of RT-associated RNase H in submicromolar concentrations and DNA-dependent DNA-polimerase activity in low micromolar concentrations. The nucleotide sequence of the inhibitors had no significant impact on their
capacity to inhibit both RT and IN. In addition, the inhibitors were still active against several RT mutants possessing resistance to non-nucleoside inhibitors (K103N/Y181C, V106A, Y188L). The ability of mODNs conjugates with eosin to block a direct binding of RT and IN was estimated. Thus we can consider these compounds as inhibitors of a new generation, capable to suppress the activity of two viral enzymes acting at early steps of HIV-1 infection and the interaction between them.

The work was supported by RFBR (grants 11-04-01004 and 12-04-31630-mol_a).

**SW01.W5–22**

**The study of 5-fluorouracil activation and distribution in lymphocytes and blood plasma**

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Inside the cell cytostatic 5-fluorouracil (5-FU) is transformed to its active form – 5-fluorodeoxyuridine. Intensiveness of 5-FU activation depends on its influx and the presence of ribose or its sources.

**Aim:** The present study was the investigation of 5-FU activation in the presence of thymidine as a ribose donor and the influence of membrane effects on this process.

**Materials and Methods:** The whole venous blood of 10 healthy volunteers (five men and five women aged from 18 to 37 years-old) was used for experiment. Lymphocytes were separated from blood and resuspended in autoplasm.

First series of experiment included incubation of samples with 5-FU (1.7 mM) alone under the 37°C for 3–60 min and further isolation of lymphocytes from plasma. The 5-FU concentration in lymphocytes and plasma was measured using HPLC.

For the second series of experiment one half of resuspended lymphocytes was twice frosted for membranes destruction, and the second half was used without cells damage. Samples were incubated under the 37°C for 3–30 min with the mixture of 5-FU (1.7 mM) and thymidine (1.7 mM) and the concentration of 5-FU, 5-fluorodeoxyuridine, thymine and thymidine was determined using HPLC.

**Results:** The intracellular concentration of 5-FU in lymphocytes already at the 3-day minute of incubation was at least 2.7 times higher (statistically significant difference at p = 0.01) than in plasma. We didn’t find any significant temporal changes in 5-FU concentration both in cells and plasma.

In both samples (with damaged and whole lymphocytes) the linear (correlation coefficient – 0.96–0.97) time-dependent increase of 5-fluorodeoxyuridine and thymine was observed. The formation of these products arises less intensively in plasma with lymphocytic extract. As 5-FU rapidly enters the cell the thymidine passage through membrane seems to be a limiting factor for 5-fluorodeoxyuridine formation.

**Conclusion:** The obtained data testify that in vitro 5-FU is rapidly accumulated in lymphocytes against its concentration gradient. Thymidine serves as a ribose source for 5-FU activation. In vitro the ability of the ribose donor to pass through lymphocytic membrane is a limiting factor for 5-FU activation.

**SW01.W5–23**

**Modified oligonucleotides as inhibitors of HIV-1 enzymes**

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Upon HIV-1 infection of a target cell, viral reverse transcriptase (RT) copies the genomic RNA to synthesize the viral DNA. Then viral integrase (IN) catalyzes insertion of the viral DNA into infected cell genome. Inhibition of the activity of these proteins should completely block vital activity of the virus right after the infection. New and effective drugs against HIV-1 continue to be required, which prompted us to search for compounds aimed at inhibiting both RT and IN. We studied the effect of short single-strand oligonucleotides conjugated with hydrophobic molecules on the IN and RT catalytic activities. Step-by-step modifications in both oligonucleotide and hydrophobic parts of the conjugate were carried out and the modification influence on the inhibitory effect was determined. Both parts of the conjugate, oligonucleotide and hydrophobic, was found to play an important role in the inhibition of both RT and IN. The oligonucleotide conjugates with 2, 4, 5, 7-tetrabromofluoresceine (eosin) were found to be the most efficient inhibitors of IN (IC50 = 50 nM); they also inhibited the activity of RT-associated RNase H at submicromolar concentrations. The inhibitors were still active against several RT mutants possessing resistance to non-nucleoside inhibitors (K103N/Y181C, V106A, Y188L) and IN mutants resistant to the strand transfer inhibitors (E92Q, G140S/Q148K and N155H). The ability of the conjugates to block a direct binding of RT and IN was also studied. Being delivered intracellularly using a cell-penetrating-peptide, the conjugates were found to impede HIV-1 replication at nanomolar concentrations. Extensive analysis showed that viral cDNA synthesis was severely impaired by mODNs.

The work was supported by RFBR (grants 11-04-01004 and 12-04-30-mol_a).

**SW01.W5–24**

**Investigation of p53 codon 72 polymorphism and cytotoxicity in HEPG2, MCF-7 and HEK293 cell lines**

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Apoptosis is the programmed cell death which has functions in elimination of unwanted tissues, remodelling of the tissue at the embryogenesis phase, in provision of development and homeostasis in later years, in aging process, in control of damaged or transformed tissues and when healthy tissues come to the end of their life. Therefore apoptosis acts as a guardian against cancer by eliminating transformed or potentially harmful cells. In this study, liver cancer cell line (HEPG2), mammary cancer cell line (MCF-7) and healthy human embryonic kidney (HEK293) cell lines were used with the aim of examining the apoptotic-anti-apoptotic mechanisms which are defective in cancer cells. We aimed to trigger apoptosis in two different cancer cell lines and one healthy cell line by the treatment of one of the most potent anti-cancer drug, Doxorubicin (DOX) and to investigate the polymorphism of p53 gene which has a major role in apoptosis. In order to achieve this, all of the cell lines were treated with the increasing doses (50, 100, 250, 500, 750, 1000, 1500, 2000, 3000 and 4000 nM) of DOX. MTT cytotoxicity assay was performed to determine the most effective dose of DOX. It was observed...
that 3000 nM is the most effective dose to display cytotoxicity. The viability of the cells were decreased significantly at this concentration; we have observed 74% and 58% decrease in viability in HEPG2 and MCF-7 cells, respectively. The genomic sequence 72nd codon polymorphism of p53 was investigated by using restriction enzymes, BstFnl and FnnDII. In both cancer cell lines, heterozygote Arg/Pro genotypes were identified in 72nd codon polymorphism as in both cell lines, 72nd codon encodes Arginine amino acid.

### SW01.W5–25

**Capsaicin induced apoptosis and gene expression dysregulation of human acute lymphoblastic leukemia CCRF-CEM cells**

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**Background:** Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) which is an ingredient of red chili pepper consumed as spice throughout the world is defined as a ‘double-edged sword’ agent for having possible tumorigenicity and genotoxicity properties, for being antagonist to carcinogens and mutagens as well as for inducing tumor cell apoptosis. However, the reasons of capsaicin induced leukemic cell apoptosis are still being investigated. In this study, we aimed to determine the effects of capsaicin treatment on proliferation and gene expression profiles of acute lymphoblastic leukemia cell line CCRF-CEM.

**Materials and Methods:** Cell viability was assessed by WST assay and IC50 dose of capsaicin was determined. After 72 h of capsaicin treatment with IC50 dose, total RNA was isolated, reverse transcribed with the transcriptor high fidelity cDNA synthesis kit and target genes’ expression levels were determined by real time qRT-PCR using the LightCycler 480 instrument. Statistical analyses of gene expressions were calculated by CLC Main Workbench software. After normalization to the housekeeping genes, log2 transformation was performed to the expression values and fold changes were calculated.

**Results:** Capsaicin inhibited the growth and proliferation of CCRF-CEM cells in a dose dependent manner. Increased mRNA expressions of caspase gene family members, activated caspase-3 and decreased mRNA expression of BCL-2 gene indicated that the apoptotic response to capsaicin. Furthermore, capsaicin treatment suppressed the key cell signaling pathway members (KRAS, AKT, GAB2, PTPN11, BRAF, INPP5D, MAPK7 and MTOR) expression levels significantly.

**Conclusion:** Our data show that capsaicin induces apoptosis in CCRF-CEM cells and this response is associated with triggering the apoptosis and down regulation of cell signaling pathways. Therefore, the possibility of capsaicin to take place in the treatment of acute lymphoblastic leukemia as well might create a new initiative in therapeutic application area.

### SW01.W5–26

**Inhibition of c-Myc transcription by olivomycin a involves preferential drug binding to NFAT/Sp1 promoter site**

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The aureolic acid derived antibiotics, that is, mithramycin, chromomycin A3 and olivomycin A, exert potent cytotoxic effects and are investigated as putative anticancer drug candidates. These compounds bind to GC-rich stretches in the DNA minor groove, thereby altering DNA conformation and interfering with template syntheses. The site of binding of Sp1, a transcriptional factor known to interact with GC-rich sites in the gene promoters, has been identified as a target for anti-transcriptional effect of mithramycin since this agent bound to this site and attenuated Sp1-dependent transcription. Recently we demonstrated that olivomycin A (1) at low micromolar concentrations rapidly inhibited mRNA levels of dozens of genes in human colon cancer and leukemia cell lines. c-Myc gene transcription was a major target of this effect of 1, with a approximately 50-fold decrease of c-Myc mRNA in cells exposed to 1 for 3–24 h. To get insight into the mechanisms of c-Myc down-regulation by 1 we focused on the GC-enriched nucleotide region 5’ from the transcription initiation site of the c-Myc gene recently shown to be the binding site for nuclear factor of activated T-cells (NFAT) and Sp1 transcriptional factors (NFAT/Sp1 site). Herein we show that NFAT/Sp1 site (dTGGCGGGAAAAAGGACTG) is preferential for binding of 1 to DNAs of various nucleotide content. Indeed, the association constants (K_a) of 1 to double stranded DNA and to NFAT/Sp1 duplex were 3 x 10^9 and 6 x 10^8M, respectively. Importantly, the GC-AT base pair (bp) substitutions (G7/C7/ T3:A3) in the NFAT/Sp1 duplex significantly decreased the association constant (10^7M) and further sequence (G7/A3) changes result in no specific binding. Interestingly, high affinity complexes have an increased quantum yield of fluorescence as well as longer fluorescence lifetime indicating an important role of nucleotide environment for drug-DNA interaction. The high K_a values determined at equilibrium are in line with the kinetically defined K_a. The association-rate parameters obtained by stopped-flow kinetics showed that the mechanism of olivomycin A:NFAT/Sp1 binding occurs via two steps, one fast and much slower one. It is noteworthy that the rates for both steps strongly depend on DNA sequence, since even unsufficient changes in nucleotide result in dramatic decrease of all binding parameters. Altogether, our data revealed that the NFAT/Sp1 binding site is a preferential target for olivomycin A.

### SW01.W5–27

**Recruitment of a phage site-specific recombinase for human gene therapy**

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The lambda-like coliphage HK022 site-specific recombination system has advantages over other site-specific recombination systems in the potential applications for gene therapy. That is because its attB recombination target of 21 base pairs is the shortest known. We have identified in the human genome secondary attB sites in the vicinity of several deleterious human mutations that resemble attB and are recognized by the wild type Integrase (int) recombinase of phage HK022. Such secondary attB sites are potential substrates for productive Int-catalyzed RMCE (recombinase-mediated cassette exchange) reactions to cure hereditary disease.
SW01.W5–28
New method for mismatch detection by means of oligonucleotide-nanogold chimeric probes
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Oligonucleotide nanostructures are of great interest for the development of nucleic acids analysis methods. These structures are usually covalent conjugates of oligonucleotides with nanoparticles such as gold nanoparticles or quantum dots. Among the problems of current molecular biology and genetics the important place belongs to the analysis of single nucleotide polymorphisms (SNPs). Today many SNP-genotyping methods were proposed but it seems the most convenient methods based on direct accumulation of analytical signal while operating with single copies of nucleic acids molecules.

We optimized the colloidal gold synthesis protocol for further immobilization of modified oligonucleotides. Chimeric oligonucleotides (DNA-RNA-DNA) with a spacer moiety and mercapto group at the 5′-end and an amino group at the 3′-end of the hairpin structure were synthesized. The sequences of probes were selected for SNP detection in rs13280 (Homo sapiens, chromosome 8). The resulting modified oligonucleotides were involved in conjugation reaction with gold nanoparticles followed by purification of nanoprobe. Nanoprobes were successfully tested in cycling probe technology previously described [1].

Reference

SW01.W5–29
Optimisation of circulating cell-free DNA (cfDNA) purification for KRAS mutation and HPV detection in cancer patients
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The presence of circulating cell-free DNA (cfDNA) in blood may have a potential application for molecular diagnosis. The purpose of our research was to elaborate the highest recovery of cfDNA for HPV16 detection and KRAS mutation using the TaqMan technology. The concentration of cfDNA was measured using a TERT gene as a marker for the total amount of cfDNA.

CIDNA from the plasma of a KRAS-positive patient was purified using the four kits listed here: QIAamp DNA Blood Mini, QIAamp Circulating Nucleic Acid, Sherlock AX and Genomic Mini AX Body Fluids. The lowest concentration of cfDNA was obtained using the Blood Mini kit (2.85 ± 0.28 ng/ml). The remaining three purification processes gave a concentration range of 15.44 ± 2.81–18.05 ± 8.89 ng/ml. The p.G13D mutation of KRAS was detected in all three purifications (Ct range 36.2–37.6), but not identified in cfDNA purified by the Blood Mini kit. Before the above-mentioned processes we had carried out other comparable experiments with similar results, showing that the Blood Mini kit results were consistently low. In the next analysis we left out the Blood Mini kit because it created a lower concentration of cfDNA. Therefore in this analysis we compared the three remaining kits. The median values for the total concentration of cfDNA were as following: 6.78 ng/ml for the Body Fluids kit; 5.38 ng/ml for the Sherlock kit and 7.39 ng/ml for the Circulating kit. There were significant differences in cfDNA concentration which we got from the Body Fluids kit and the Sherlock kit (p = 0.04). Since the Sherlock kit is manufactured to isolate DNA from small volume samples, and it gave the lowest cfDNA concentration, the two remaining kits were used in the next experiment regarding the effectiveness of HPV16 detection. When 1 ml of plasma was taken the mean concentration of cfDNA was 11.40 ± 2.0 ng/ml. When 0.2 ml was taken the mean concentration of cfDNA was 15.20 ± 4.3 ng/ml. However, the isolation from 1 ml gave us the possibility to detect HPV16 in a final volume of 100 µl of plasma (per 1 PCR) with a notably earlier Ct (range 29.1–29.5).

In conclusion, the best purification of cfDNA was obtained using the Genomic Mini AX Body Fluids and QIAamp Circulating Nucleic Acid kits. However, the Genomic Mini AX Body Fluids kit gives us the possibility to enlarge the final volume of plasma up to 500 µl for future analysis.

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SW01.W5–30
Nucleic acid aptamers against Plasmodium lactate dehydrogenase for malaria diagnosis – discovery, characterization, structure and application
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Better ways to diagnose malaria at the point of care are critically needed. We have been developing DNA aptamers against malaria diagnostic targets including Plasmodium lactate hydrogenase for new diagnostic devices. Here, we report the discovery of aptamers against lactate hydrogenase that are highly specific for the Plasmodium lactate dehydrogenase with binding affinities in the nanomolar range as determined by isothermal titration calorimetry and by other techniques. Due to a counterselection aptamer selection strategy, the aptamers do not bind to human lactate dehydrogenase. We successfully solved the X-ray crystal structure of one aptamer in complex with its target, revealing an unusual DNA fold that explains the nature of the specificity of the binding interaction. The aptamers were coupled to nanoparticles to develop a colorimetric sensor. The aptamers are being further developed using a number of different strategies to develop a malaria diagnostic test suitable for clinical trials. This new structure of a novel and important aptamer-target pair will be an important milestone in developing diagnostic applications of DNA aptamers in future.

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SW01.W5–31
Quantitative imaging analysis of replication stress in cells exposed to DNA targeting anticancer drugs and oxidative stress
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Background: A recently described method (Berniak et al., submitted) of quantitative analysis of spatial (3D) relationship between discrete nuclear events detected by confocal microscopy was applied in analysis of a dependence between sites of DNA damage signaling (γH2AX foci) and DNA replication (EdU incorporation) in cells subjected to treatment with topoisomerase inhibitors camptothecin (CPT), etoposide (ETP), mitoxantrone (MTX), cis-platinum (CS) or hydrogen peroxide (H2O2).

Methods: Newly synthesized DNA was fluorescently labeled using a precursor EdU (5-ethynyl-2′-deoxyuridine) and a ‘click’ reaction. γH2AX foci were labeled by immunofluorescence. Images of replication and histone H2AX phosphorylation sites were recorded using confocal microscopy aided with deconvolution. Nearest-neighbor and correlation analyses were performed using an algorithm written specifically for this task.

Results: CPT induces γH2AX foci, likely reporting formation of double-strand DNA breaks (DSBs), almost exclusively at sites of DNA replication. ETP induces γH2AX in S-phase, with a moderate tendency toward replication sites. MTX induces γH2AX foci with no detectable preference for replication foci. Histone H2AX phosphorylation induced by CS is detected 2 and 4 h after exposure to the drug and largely coincides with replication foci. Oxidative stress leads to induction of γH2AX in replicating as well as non-replicating cells, and only a weak tendency toward damage at sites of DNA replication.

Conclusions: High degree of colocalization of EdU and γH2AX sites in cells treated with CPT is coherent with the known mechanism of induction of DSBs by DNA topoisomerase I (topo1) inhibitors at sites of collision of moving replication forks with topo1-DNA ‘cleavable complexes’ stabilized by CPT. The moderate or poor correlation of replication sites with γH2AX foci as seen in the case of ETP or MTX (also observed with these drugs in non-replicating G1 and G2 cells), indicates on the mechanism of DNA damage unrelated to replication. The increased number of replication foci observed in cells treated with CPT suggests that stalling replicating forks may trigger activation of new DNA replication origins. This agrees with a postulated plasticity of replication origins.

References

SW01.W5–32
Mechanisms in biology via ‘cardiolipin machinery’: cardiolipin-induced DNA assembly
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Nuclear lipids play an important role in chromatin function and organization. Cardiolipin (CL) level is dramatically increased in cells with active chromatin [1]. Furthermore, dissociation of CL-cytochrome c complex leads to apoptosis initiation. Keeping in mind a possible key role of cardiolipin in chromatin function and in apoptosis, we studied an interaction between polydeoxyribose nucleotides (PN) and cardiolipin by a variety of methods: HPLC chemo sensor, atomic force microscopy (AFM), circular dichroism (CD) and spectrophotometry (UV). We used poly(dA).poly(T), poly(dG).poly(dC) and poly(dAT).poly(dAT) and poly(dG).poly(dG) as DNA. It was found that complexes between PN and CL are characterized with different chromatography peaks compared to initial PNs. Crystals of DNA-CL aggregates are bigger than DNA AFM images (typing mode). It was shown that CL can interact with DNA of various AT/GC content [2]. DNA molecules are known to form liquid-crystalline structure (LC) in the presence of polyethylene glycol (PEG). Such structures are characterized by high intensity negative bands at their CD spectra (α, 150). But DNA solutions, containing CL, demonstrated lower CD effects. Thus, complexation with CL prevents DNA molecules from forming of PEG LC structure. In opposite, CL has no effect on the preformed DNA LC structures. Titration of DNA PN with CL leads to shift of band in UV adsorption spectra from 260 to 258.3 nm for poly(dA) poly(dT), which testifies CL – DNA complexation [3]. The results presented can be discussed in a frame of ‘cardiolipin machinery’ conception on involving of CL – DNA/protein/membrane interactions into general biological mechanisms while each CL molecule interacts with either two DNA molecules, or DNA and protein or DNA and membrane.

References

SW01.W5–33
Ku protein as an intracellular target of extracellular DNA
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Biological effects of extracellular DNA (exDNA) suggest its interaction with the effector molecules exposed on the cell surface or localized inside the cell. We have earlier demonstrated that substantial amount of exDNA is bound to the cell surface (csbDNA) via DNA–protein interactions and this DNA inhibits polyIC-induced production of proinflammatory cytokines in the human umbilical vein endothelial cells (HUVEC) [1]. The goal of this work was to identify the cellular targets of immunoinhibiting csbDNA using ODNs containing the nucleotide motifs frequently found in csbDNA and displaying the same effects.

The binding of [32P]-labeled single- and double-stranded ODNs (ss- and ds-ODNs) with membrane-cytosolic extracts and living human umbilical vein endothelial cells (HUVEC) was studied by electromobility shift assay (EMSA). Complexes of biotinylated ODNs with target proteins were affinity isolated using streptavidin sepharose with subsequent SDS-PAGE and identified by MALDI-TOF mass spectrometry.

Both ss- and ds-ODNs form strong ODN–protein complexes with similar electrophoretic mobilities after incubation with the membrane-cytosolic extracts of HUVEC either when added extracellularly or lipofected into cells. ODN-binding proteins were identified as DNA-binding components of DNA-dependent
protein kinase (DNA-PK), namely, Ku70 and Ku80 proteins. Diverse cellular localization and functions of Ku protein demand further clarification of Ku70/80 role as a mediator of the eubDNA immunoinhibiting effects.

Reference

SW01.W5–34
DNA as a target for anticancer drugs based on the coordination compounds of metals
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DNA interaction with coordination compounds of platinum, palladium, ruthenium was investigated in water solutions with different NaCl concentration. Mono- and bi-platinum compounds, different ligands in the coordination sphere of complexing ions of palladium and ruthenium were regarded. It was shown that the coordination bonds between metal ions and DNA bases are usually typical for anticancer agents of this class, but connected with the central ion ligands can modify greatly the biological activity of compounds. The inclusion of antibiotics, radioprotective and radiosensitizers into the first coordination sphere of the ion (or as side ligands) may change the way of complex ion binding to DNA as well as may influence on the cumulative therapeutic effect of treatment. The molecular model of DNA interaction with different compounds was constructed on the base of experimental data of different methods: spectral (electronic spectra, fluorescence, circular dichroism), viscometric, dynamic light scattering, flow birefringence, gel electrophoresis, atomic force microscopy. The comparative analysis of DNA interaction with a compound and its components provides an opportunity to highlight the role of the individual groups of compounds under study in binding. The possibility of the construction of multicomponent systems with the inclusion of metal nanoparticles and nanoclusters is also under consideration. The application of DNA-organo-metallic complexes in new technologies was discussed. For example, DNA linkage with the coordination compounds of any metals with the different components - fluorophores, aromatic ligands, ready - for-enhancement effect nanoparticles can produce the original way for the creation of new materials for medical and nonmedical use.

SW01.W5–35
An algorithm for multiparameter cytometric analysis of spatial relationships between nuclear events represented by microfoci
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Background: Laser scanning cytometry (LSC) studies of cell populations have demonstrated that the level of DNA damage induced by topoisomerase I inhibitor camptothecin, or oxidative damage induced by H2O2 is higher in cells replicating DNA than in nonreplicating G1 and G2 cells. In order to establish a potential interdependence between replication and damage, it was necessary to analyse quantitatively the distances and correlations between replication and DNA damage foci in 3D space, in large number of nuclei.

Goal: This work was focused on constructing an algorithm for quantitative analysis of spatial relations between two classes of discrete events represented by large numbers of foci in three-dimensional images of cell nuclei.

Methods: Newly replicated DNA was labelled using incorporation of a precursor (EdU) and click chemistry; foci of phosphorylation of histone H2AX (a marker of double strand breaks) were labelled by immunofluorescence. 3D confocal fluorescence images were deconvoluted and subjected to image analysis.

Results: An algorithm for quantitative analysis of spatial relationships between discrete events (microfoci) in cells was constructed and applied in analysis of a relationship between DNA damage signaling (H2AX histone phosphorylation), and DNA replication in nuclei of cells treated with camptothecin or hydrogen peroxide. The relationship between the spatial distributions of these two signals was analyzed using nearest-neighbour (nn) distance to isolate the populations of replication-dependent and replication-independent damage signals. The nn distance calculation was supplemented with analysis of cumulative distribution of all possible distances (Ripley’s K functions) between signals of the same and different kinds.

Conclusions: We found an expected, statistically significant spatial correlation between replication and damage induced with topoisomerase I inhibitor, but only a negligible correlation in cells subjected to oxidative damage. This approach to analysis of spatial association of two nuclear events is expected to be suitable for investigations of a relationship between any other types of cellular events represented by small foci, in multicolor patterns found in 3D confocal images.

SW01.W5–36
New mechanism of genetic control: nature and properties of DNA-bound lipids
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Since DNA-bound lipids were discovered in the chromatin of a number of eukaryotic and prokaryotic cells, we performed a study of such of DNA-lipid complexation by biochemical, biophysical and molecular docking/dynamics approaches to clarify the mode of DNA-lipid interactions, DNA-lipid recognition phenomenon and to determine the structural parameters of lipids in genomic DNA. Studies with synthetic DNA polynucleotides revealed that neutral lipids (oleic acid and cholesterol) can be tightly bound to DNA double helix (Zhdanov et al., 2002). Whereas duplexes are influenced by oleic acid ligandation, which could not be removed by the ethanol dialysis procedure, no binding occurs to triple stranded DNA. The spectroscopic results indicate that oleic acid shows molecular recognition to AT b.p. motifs by groove binding. CG tracks – in particular, alternating d(CG) motifs – are strongly influenced by ligand interaction up to a ratio of one molecule per two base pairs. As a consequence, a new mechanism of regulation of gene expression at the nuclear membrane or by lipids inside DNA double helix has to be discussed. It could be suggested that fatty acid (oleic acid) binding to d(CG) tracks in an ‘exhaustive’ type represents a possible mechanism for a stabilization of CG-dimucleotides repeats in non-coding area of human genome. Recognition mode for oleic acid – poly d(A)poly (T) suggests, likely, fatty acid molecule binding to the minor groove, which is more hydrophobic. Since
there is lipid fraction in nucleus, tightly bound to DNA (Zhdanov et al., 2006), we postulate an existence of new regulation and informational level at genomic DNA: lipids specifically bound to genomic DNA depending from nucleotide sequence, namely, lipid code of genomic DNA.

References

SW01.W5–37
The eNOS 4a/b gene polymorphism as a genetic marker of aging in Kazakhstan population
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Aging is characterized by development of oxidative stress. Genes involved in the oxidative stress regulation can play a key role in development of age associated pathologies and susceptibility to the active longevity. Strong evidence suggests that nitric oxide (NO) plays important role in adaptation to oxidative stress and in development of age associated disease. Nitric oxide is synthesized by endothelial nitric oxide synthase (eNOS) and data show that the polymorphism of eNOS signiﬁcantly affects concentration of NO in blood plasma. In our study we tested the hypothesis whether the eNOS VNTR 4a/b gene polymorphism is a genetic marker of aging in Kazakhstan population.

The eNOS VNTR 4a/b gene polymorphism was tested in 338 inhabitants of Almaty City: 94 subjects with age-associated diseases (cardiovascular disease, cancer, arthritis, cataracts, osteoporosis, type 2 diabetes, hypertension, etc.) and 244 subjects with no history of age-associated diseases. Genotypes were determined by polymerase chain reaction. The frequencies of 4a/b genotypes in patients with age-associated diseases and control group were as follows: in patients the frequencies of the 4aa, 4ab, or 4bb genotype were 5.3%, 23.4%, or 71.3%, respectively, and in controls the genotype frequencies were 0.8%, 12.7%, or 86.5%. In this study intron 4aa genotype (allele for four repeats of 27 bp) of eNOS gene was associated with CRC in population from Kazakhstan. The preliminary analysis demonstrated that the eNOS VNTR 4a/b gene polymorphism is a genetic marker of aging in Kazakhstan population.

study of the association between GST-deletions and susceptibility to CRC in population from Kazakhstan are presented here.

The peripheral blood samples were taken from 191 CRC patients and 191 healthy donors, residents of Almaty city and surrounding region. The control group of healthy people was chosen according to the age, ethnicity, gender and smoking habits of CRC patients.

GSTM1 and GSTT1 alleles were genotyped by the multiplex PCR method. The distribution of GST-genotypes in CRC cohort differs from control cohort. However both cohorts show the correspondence to the Hardy-Weinberg equilibrium. Among the CRC patients and healthy persons we defined the carriers of the following GSTT1-genotypes: ‘-/-’ – 39.8% and 60.2%, ‘+/-’ – 30.9% and 15.7%, ‘+/+’ – 29.3% and 24.1%, respectively. The GSTM1 genotypes distribution among people suffering from CRC and control group members was followed: ‘-/-’ – 36.6% and 49.7%, ‘+/-’ – 13.1% and 16.2%, ‘+/+’ – 50.3% and 34.0%, respectively.

The statistical analysis of association between the GST-polymerophism and susceptibility to colorectal cancer were done. Data show that loss of both GSTT1 and GSTM1 functions can predispose to the colorectal cancer development. The increased risk of CRC was determined for the GSTT1 (-/-) homozygous and heterozygous (+/-): OR = 2.29, \[ \chi^2 = 15.93, \text{ CI} = (1.52–3.45), \] p < 0.001, according to the dominant model of inheritance. The carriers of GSTM1 deletions in homozygous state (-/-) also show the strong associations with susceptibility to CRC: OR = 1.96, \[ \chi^2 = 10.40, \text{ CI} = (1.30–2.96), \] p < 0.006, according to the general model of inheritance.

So, the structural deletions of GSTT1 and GSTM1 genes which lead to the loss of corresponding enzymes functions can increase the relative risk of colorectal cancer development in populations from Kazakhstan.

SW01.W5–38
Loss of functions of glutation S-transferases can predispose to colorectal cancer
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Glutathione S-transferases (GST) are involved in detoxiﬁcation of many potentially carcinogenes. The deletions of GSTT1 (0 class) and GSTM1 (µ class) genes show the associations with many cancer types. Few studies have evaluated the relationship between deletion polymorphisms of GSTT1, GSTM1 genes and the increased risk of colorectal cancer (CRC). The result of first
whole structure is folded and cation capture there is no signifi-
cat difference between groups in simulation.

During simulations with cation capture, seven groups showed
low stability during cation absence in central cavity. Thus, these
groups loop topology negatively affects on G-quadruplex struc-
tural stability. These groups contain propeller and/or lateral
loops or no loops at all. The rest three groups, bearing diagonal
or combination of diagonal and lateral or diagonal and propeller
loops, turned out to be rigid. Diagonal loops presence allows
longer existence without an ion and assists its capture and reten-
tion inside.

In conclusion we may range quadruplex stability in folowing
way: Diagonal loops > Lateral loops > Propeller loops > No
loops. The most stable quadruplexes in our selection turned out
to be artificial, natural ones turned out has intermediate stability.

SW01.W5–40
Genes encoding RNA binding proteins / RNA
splicing factors as potential therapeutic
targets in heart failure in dogs
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RNA-binding proteins are involved in alternative mRNA splicing
which contributes to the total complexity of the transcriptome
and proteome. Aberrant expression of splicing regulatory factors
has been implicated in a number of clinical disorders [1]. Yet, lit-
tle is known about the scope of alternative splicing at a whole
genome level in heart diseases. The aim of the present study was
to investigate whether the expression of genes involved in mRNA
splicing is changed in the blood of dogs suffering from heart dis-
ease (HF).

The study was conducted on 37 dogs. A clinical examination,
ECG, EGC and thoracic X-ray were carried out. The dogs were
divided into groups according to ISACHC classification
(ISACHC Class I: 13 dogs, ISACHC Class II: 13 dogs). A con-
trol group consisted of 11 healthy dogs. Total RNA was
extracted from whole blood samples and the transcriptomic pro-
file analysis of peripheral blood nuclear cells was performed using
microarray technology (Agilent, USA). The results were analysed
with Gene Spring Software

The study revealed 1580 differentially expressed transcripts
between healthy dogs and class II heart failure; and 5685 differ-
entially expressed transcripts between ISACHC I and II groups
(student’s t-test, FDR<0.05). Among these two differentially
expressed sets there were 1127 common transcripts. An analysis
using Panther database revealed that 94 of them encode proteins
from RNA binding class. Moreover, there were 30 genes which
molecular function is RNA splicing factor activity including
SRSF3 Canisfamiliaris splicing factor gene whose product has
been recently proposed as a target of a cardiotonic steroid – digi-
toxin [1]. The SRSF2 gene was also differentially expressed.
Furthermore, there were two SRSF variants (7 and 11) not yet
described in relation to HF.

References
steroid digitoxin regulates alternative splicing through deple-
tion of the splicing factors SRSF3 and TRA2B. RNA 18,
1041–1049.
AVet.2012.002
The science of enzymology was created to understand the extraordinary catalytic power of enzymes, which can accelerate reaction rates by 12–15 orders of magnitude at room temperature in aqueous solution. The methods employed include sophisticated kinetic measurements, studies of isotope effects, and site-specific mutagenesis of putative catalytic residues. Computational tools incorporating both molecular dynamics simulations and quantum mechanical calculations were also created to model enzyme-catalyzed reactions. In the 1980s, the new field of structural enzymology was developed, in which crystal structures of enzyme-inhibitor complexes (and, later, actual enzyme-substrate complexes) were determined to provide clues to the detailed catalytic mechanisms. Always, these mechanisms were worked out by reference to the known behavior of simpler small molecule catalysts. Ultimately, as X-ray sources became brighter and techniques for stabilizing reaction intermediates in protein crystals were refined, it became possible to observe many enzymes in action at true atomic resolution, something not possible for the more complicated small molecule catalysts typically employed by industry and synthetic organic chemistry. This project has its origins in our realization that enzymology as currently practiced has techniques more powerful than those available to study non-enzymatic organic and organometallic catalysts, and consequently enzyme-catalyzed reactions are actually better understood in many cases. We hypothesized that, if we incorporated poorly-characterized small molecule catalysts into easily-crystallizable proteins, the powerful techniques of mechanistic and structural enzymology could then be applied to these systems as well. We call this new field ‘reverse enzymology’, because it reverses the typical enzymological paradigm of using insights from small molecule catalysis to understand the behavior of enzymes. In this lecture, the rationale behind this new field will be explained in detail, and some examples will be given of its potential for providing new insights into the structures and mechanisms of organic and organometallic catalysts.

**References**


**SW02.S6-3**

*Some like it acid*: how *Escherichia coli* glutamate decarboxylase controls its intracellular activity in response to acid stress

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Glutamate decarboxylase (Gad; EC 4.1.1.15) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme which converts glutamate to GABA with concomitant proton consumption and CO2 release. In E. coli there are two Gad isoforms, GadA and GadB, which share 99.3% sequence similarity and are biochemically indistinguishable. Both isoforms have an acidic pH optimum, are hexamers and major structural components of the glutamate-dependent acid resistance system, which efficiently protects orally-acquired bacteria from the extreme acid stress encountered during transit through the host stomach (1).

In collaboration with Dr. G. Capitani (PSI Villigen, CH), my group has intensively studied E. coli GadB. The comparison of the crystal structures at pH 4.6 (active form) and 7.6 (inactive form) provided evidence that the pH change causes three major structural reorganizations. The first 15 residues of the N-terminal domain are required at acidic pH for recruitment of GadB to the membrane and for binding of chloride ions (2,3). The last 15 residues of the C-terminal domain are ordered only at neutral pH, when they plug the active site funnel. In particular the side chain distal nitrogen of His465, the penultimate residue in sequence, participates to the formation of a reversible covalent bond with the PLP-Lys276 Schiff base (3). Indeed His465 has a massive influence on the equilibrium between active and inactive forms (4).

In this lecture, I will discuss the evidence for a generally accepted mechanistic proposal in which decarboxylation proceeds through a metal-bound radical anion intermediate [5, 6]. In addition, a series of site-directed mutagenesis and kinetic experiments will be reported that examine (i) how a critical interaction between the conserved side chains of Trp-132 and Glu-101 (a metal ligand) controls manganese oxidation state, and (ii) the likely role of a conformationally mobile active site loop in defining whether C-C bond cleavage proceeds via an oxidative or non-oxidative transformation [7]. The implications of these studies for our understanding of the mechanism by which OxDC catalyzes cleavage of the oxalate C-C bond will be discussed.

**References**

required for fixing in place the C-terminal tail of a neighbouring subunit at neutral pH, whereas it repositions at acidic pH.

More recently we have dissected the catalytic mechanism of GadB by mutating residues in the active site and in the β-hairpin and found that they affect cooperativity in the pH-dependent activity change and substrate reprotonation following decarboxylation, respectively. Spectroscopic and kinetic analyses of these mutants, as well as studies aimed at establishing a more precise role for GadA and GadB isofoms in the cell under stressful conditions will be discussed.

References

SW02.S6–4
Towards the understanding of age-specific regulatory variation
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A number of heritable polymorphisms, the majority of them non-coding, are associated with late-onset phenotypes. Many such polymorphisms are common as they escape the main load of purifying selection. Mechanisms underlying late-onset aberrations are likely to be diverse and potentially include epistatic buffering effects. We are taking advantage of MRC Harwell’s Ageing Mutant Screen, as well as of polymorphisms between laboratory mouse strains, to systematically profile age-specific changes in gene expression. These will then be mapped to genotypes and genomic regulatory features such as domains of histone modifications, DNase I hypersensitivity regions and transcription factor binding sites. This work will help gain mechanistic insights into the biology of age-related phenotypes and explain why such phenotypes are unobservable in the young age. The project is currently in its pilot stage and initial results will be presented here.

SW02.S6–5
Complementary role of the two metal ions in the catalytic reaction of RNase H
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Phosphate esters are essential to living systems. In a large number of enzymes, the phosphate ester hydrolysis is catalyzed by two bound divalent metal ions. We recently found that coupled proton transfer reactions emerge as central factors in the catalytic reaction, however, the exact roles of the two metal ions remains unclear. We carried out hybrid quantum-classical QM/MM free energy simulations of the catalytic reaction of the bacillus halodurans RNase H enzyme complexed with an RNA/DNA hybrid substrate. We find that the presence of both catalytic Mg-ions is an essential requirement for catalysis. Replacing either one of them with a Ca-ion abolishes the catalytic activity. Double Mn- or Ca-ion replacements have been characterized experimentally and our simulation results agree with measured catalytic activities. However, targeted single ion replacements have only been possible in our simulations. We identified that the main role of the metal ions is to decrease the pKa of the oxygen nucleophiles and, therefore, to decrease the barrier of the coupled proton transfer processes governing the catalytic reaction. Our proton transfer mechanism, together with the corresponding highly accurate transition state structure, represents an excellent target for structure-based drug design studies of novel HIV-specific inhibitors.

SW02.S6–6
Kinases, phosphatases, mutases and G-proteins
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Using a combination of multinuclear NMR spectroscopy, high resolution X-ray crystallography, synthetic chemistry and computational chemistry we have explored the conformational behaviour of proteins under a very wide range of conditions. Recently, we have focussed on enzymes that catalyse the transfer of phosphoryl groups, where the non-catalysed reactions can be among the slowest known for a physiological process: phosphate monoesters, for example, have calculated lifetimes to spontaneous hydrolysis of up to 1012 years. I will use a range of phosphoryl transfer enzymes to illustrate what contributes to the very high levels of catalysis achieved by these enzymes. Specifically, we have examined what happens during domain folding, during assembly of the native enzyme, during substrate binding, and during transition state binding. The introduction of metal fluoride species to mimic the ground state and the transition state of the transferring phosphate group has allowed us to dissect the steps involved in catalysis. In particular, these enzymes illustrate how the charge distribution in the close vicinity of the transferring phosphate is tightly controlled by the enzyme, and how the near transition state complex conformation reacts to modulation of these charges. I will also discuss phosphoryl transfer in the context of the domain closure required to bring about catalysis.

References

SW02.S6–7
New insights in HIV protease substrate and inhibitor binding, studying by fast kinetic approach
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HIV-1 protease (PR) remains an important pharmaceutical target. Understanding of the mechanistic events associated with the binding of HIV-1 PR substrates and inhibitors is crucially important for the design of novel inhibitors of the enzyme. At present time there are a lot of crystal structures of substrate- and inhibitor-bound PR, NMR studies and molecular dynamics simulation studies. These data provided relevant information insight into the molecular mechanism of PR action and resistance to inhibitors. The fast kinetic approach allows to detect and analyze the rates of formation/decomposition of transient complexes during interaction of enzyme with substrate and inhibitors. Therefore, kinetic study offers a good opportunity for understanding the drug resis-
Purification and characterization of permuted penicillin acylase from Alcaligenes faecalis

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Penicillin acylase (PA; EC 3.5.1.11) catalyzes the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid. PA is widely used in pharmaceutical industry for production of semi-synthetic penicillins and cephalosporins and in fine organic synthesis for preparation of chiral compounds.

In the case of PA the majority of publications were devoted to the enzyme from Escherichia coli (EcPA). However, PA from other bacterium, Alcaligenes faecalis, is superior to EcPA in many characteristics. In our laboratory new unique gene of PA from A. faecalis VKM B-1518 (AIPa) was cloned and overexpressed in E. coli.

PA belongs to the superfamily of the N-terminal nucleophile hydrolases and comprises catalytic serine at the N-terminus of the β-subunit. Gene pac encodes inactive precursor composed of signal peptide, α-subunit, spacer and β-subunit. Precursor undergoes complicated multistep posttranslational modification to form active αβ-heterodimer.

In our laboratory protein engineering experiments with PA are carried out. It was found that in many cases single amino acid changes affect the maturation of the mutant propeptide to the active PA. Actually in the case of majority mutants, preparation of active mutant enzyme required separate optimization of cultivation conditions (including growth medium) of recombinant strain. Multistep maturation of PA could be avoided by creation of permuted enzyme gene which will encode single-chain polypeptide with catalytic Ser residue at N-terminus. That enables to simplify process of enzyme formation due to the absence of processing. The aim of our work was creation and characterization of permuted PA from A. faecalis (pAIPA).

Analysis of AIPa three-dimensional structure indicates that N-terminus of α-subunit and C-terminus of β-subunit are situated in close proximity. That fact makes it possible to connect these termini by linker peptide. We carried out computer modeling of pAIPA structure with different linkers and two best candidates were selected.

Polymerase chain reaction was used to construct pAIPA gene. Then pAIPA was expressed in E. coli as soluble and active enzyme. Optimization of cultivation conditions resulted in 15-fold increase of pAIPA yield. As well as standard purification scheme for wild-type AIPa did not allow obtaining homogenous preparation of pAIPA, the new procedure of pAIPA purification was developed. Investigation of the properties of pAIPA showed similar values of catalytic parameters compared with the wild-type AIPA, but revealed approximately 10-fold higher thermal stability of pAIPA. Thermal inactivation kinetics of wt-AIPa and pAIPA were studied in wide range of different conditions (temperature, pH, buffer type and ionic strength).

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References

**SW02.S6–10**

**Molecular recognition and regulation of human angiotensin-I converting enzyme (ACE) activity by natural inhibitory peptides**


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Anglesintin-I converting enzyme (ACE), a two-domain dipeptidylcarboxypeptidase, is a key regulator of blood pressure as a result of its critical role in the renin-angiotensin-aldosterone and kallikrein-kinin systems. Hence it is an important drug target in the treatment of cardiovascular diseases. ACE is primarily known for its ability to cleave angiotensin I (Ang I) to the vasoactive octapeptide angiotensin II (Ang II), but is also able to cleave a number of other substrates including the vasodilator bradykinin and N-acetyl-Ser-Asp-Lys-Pro (Ac-SDKP), a physiological modulator of hemotopoiensis. For the first time we provide a detailed biochemical and structural basis for the domain selectivity of the natural peptide inhibitors of ACE, bradykinin potentiating peptide b and Ang II. Moreover, Ang II showed selective competitive inhibition of the carboxy-terminal domain of human somatic ACE providing evidence for a regulatory role in the human renin-angiotensin system (RAS).

**Reference**


**SW02.S6–11**

**Enzymatic characterization of two novel enzymes with enone-reductase activity**

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An approach using three-dimensional motifs reflecting specific active site arrangements (catalophores) led to the identification of two novel enzymes with enone-reductase activity: Tth-X from Thermus thermophilus and Pho-Y from Pyrococcus horikoshii. Regarding the position and nature of active site residues, they possess significant similarities with the Old Yellow Enzyme family, although neither the amino acid sequence nor their overall structure is related. Both enzymes were cloned, expressed, purified and subjected to crystallization trials. A detailed biochemical analysis of Tth-X and Pho-Y demonstrates that these two proteins have some features in common with OYEs, for example the utilization of NAD(P)H as a reducing agent or the reduction of α,β-unsaturated ketones. On the other hand several other enzymatic properties show marked differences to those of OYEs, like the significantly more negative redox potential, and the distinct preference for quinone substrates. Therefore we suggest that Tth-X and Pho-Y can be described as NADPH-dependent quinone reductases, and that the OYE-like features are side activities. In that sense, the catalophore approach has proven to be a useful tool to discover hidden enzymatic activities.

**SW02.S6–12**

**Laccase from ascomycete Botrytis aclada: effect of mutation near the T1 site on the structure and properties of the enzyme**

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Laccases are members of a large family of multicopper oxidases, which oxidize a wide range of organic and inorganic substrates accompanied by dioxygen reduction to water. They contain four copper atoms per molecule organized in three sites: T1, T2 and T3. The first step of the catalytic cycle is the electron transfer from the substrate molecule to the T1 center. It is supposed that the redox potential of the T1 copper ion influences the enzymatic reaction rate. Redox potential of the T1 center is determined by the interaction of copper ion and strong ligands, namely two histidines and cysteine (similar for all laccases), nature of the axial residues and by the structure of the second coordination sphere of the copper ion.

In this work we investigate the effect of a single mutation in the T1 site on structure, redox and catalytic properties of the enzyme. For this purpose the axial residue of the T1 copper ion – leucine – was replaced by methionine in the recombinant wild-type laccase from the ascomycete Botrytis aclada.

The catalytic properties of both forms were characterized using ABTS and 2,6-dimethoxyphenol as substrates. It was shown that the mutation reduces the catalytic activity towards both substrates but increases resistance to the inhibition of the enzyme by fluoride ions. The mutation leads to the enhancement of the thermal stability of the laccase.

The redox potential of the T1 site center of the mutant is approximately 150 mV lower than that of the wild type.

The three-dimensional structures of both forms were determined by X-ray crystallography at 1.7 Å resolution (Rf = 16.6% and 16.5% for the native and the mutant forms, respectively). Both structures are similar (r.m.s.d. by CA-atoms = 0.11 Å) with slight changes in T1 center vicinity. The distances between T1-Cu and three strong ligands (ND-His426, ND-His494 and S-Cys489) are the same in both structures. Located in the T1-copper axial position of the mutant form Met499 is closer to the copper atom than Leu499 of the wild form. The minimal distance between T1-Cu and sulfur atom of Met499 is 3.2 Å against 3.6 Å of CD-Leu499. Copper ion in the mutant form slightly displaced towards Met, as a result the distance between Cu(II) and located in the another axial position Ile491 became longer from 3.5 to 3.7 Å.

**SW02.S6–13**

**A new mechanism of acceleration of two-dimentional reactions by confining proteins to a high-binding membrane region: assembly of tenase on activated platelets**

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One of the mechanisms invented by Nature to accelerate biochemical reactions consists in the transition from a 3D space...
(reactions in solution) into a 2D space (reactions on membrane). The most striking example is the several-orders-of-magnitude-fold increase in the rate of blood coagulation reactions on the membranes of platelets. To investigate further this phenomenon, we examined binding of fluorescently labeled coagulation factors IX (FIX) and X (FX) to phosphatidylserine-exposing platelets using confocal microscopy. Our experiments showed that these factors preferentially bind to a small convex region (10–20%) of the platelet membrane.

The goal of this study was to investigate in silico the influence of such clumping of coagulation factors on the activation of FX by the tenase (complex of FIXa and FVIIIa on the platelet membrane). We have constructed and investigated a mathematical 3D model of this process using Virtual Cell software (VCell; http://www.vcell.org). The model is based on the system of reaction-diffusion equations describing formation of the tenase on the membrane from free FIXa and membrane-bound FVIIIa and consequent activation of the free FX. The deterministic modeling shows that the clumping of factors accelerates the FX activation tenfold. This value depends on the kinetic constants used in the model but is independent of the diffusion coefficients of the proteins. The stochastic modeling shows that in the case of uniformly distributed proteins one FX is activated per second, and for the case of clumped proteins about 3 FX are activated per second. These values dramatically change with the decrease of diffusion coefficients of the membrane-bound proteins and dimension of the mesh.

Here by the in silico investigation we have shown that observed in vitro confinement of coagulation factors to a small membrane region can significantly accelerate membrane-dependent blood coagulation reactions.

**SW02.S6–14**

**Exogenous NO accelerates apoptosis of human neutrophils followed phagocytosis**

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NO molecule is a key physiological intra-and intercellular mediator (‘messenger’) involved in the regulation of many physiological processes, in particular, the maintenance of vascular tone, signaling between neurons and immune response. An increasing formation of NO in inflammation was shown to modulate neutrophil functions: inhibits the production of superoxide ion in the activated cells, affects the production of pro-inflammatory factors and phagocytosis of infectious agents by neutrophils. Activated neutrophils produce significant amount of NO, which, together with its derivative – peroxynitrite, is being apoptotic factor for neutrophils.

It was shown by us that activation of neutrophils in the course of phagocytosis of opsonized zymosan (OZ) was accompanied by significant increase in NO production. NO is synthesized in vivo via enzymatic pathway using NO-synthase (NOS), which exists in neutrophils in several isoforms.

We investigated the effect of exogenous NO on the development of apoptosis by phagocytosing neutrophils. In our experiments we used 3,3-diethyl-1,2-dioxo triazened sodium (DEA NONOate) as a donor of NO. The number of apoptotic cells was assessed by determination of fluorescently labeled annexin V binding.

We have found that DEA NONOate was able to enhance apoptosis of phagocytosing neutrophils when it was added at least 10 min before OZ. Significant increase in the number of apoptotic neutrophils, up to 2–3 times was detected. Being added simultaneously with OZ, NO-donor was not effective. This fact demonstrates the impact of nitric oxide (II) mainly on early stages of phagocytosis and, accordingly, the subsequent induction of apoptosis. Acceleration of apoptosis depended on the concentration of 3,3-diethyl-1,2-dioxo triazened sodium. Five hundred micromolar DEA NONOate caused 200% increase in the number of apoptotic cells compared with a population without the addition of NO donor and by almost 700% compared to the level of constitutive apoptosis.

Acceleration of neutrophil apoptosis following phagocytic interaction with infectious agents appears essential for the resolution of infection. Phagocytosis-induced cell death (apoptosis that results from phagocytosis) contributes to neutrophil turnover at sites of infection. We have shown that phagocytosis-induced cell death is accelerated by nitric oxide.

The work was supported by the Russian Foundation for Basic Research grant 10-04-01479.

**SW02.S6–15**

**Interaction between Drosophila CENP-C and protein phosphatase 4**

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We aim to determine the precise function of the centromeric protein, CENP-C, in kinetochore function and its regulation. In characterising proteins that interact with Drosophila CENP-C, we discovered that it co-purifies with the three subunits of the phosphoprotein phosphatase 4 (PPP4) trimeric complex: Regulatory subunit 2, Regulatory subunit 3 (in Drosophila known as Falafel) and the Catalytic subunit PPI4c. In vitro and in vivo studies confirmed this interaction and proved that a short fragment in the carboxy-terminal part of CENP-C directly interacts with the amino-terminally located PH-like domain of Falafel. Falafel is therefore a bridging component that allows binding of the PPP4 trimer to CENP-C. We also found that Falafel localizes to centromeres during interphase and mitosis. RNAi-based depletion of Falafel in cultured cells has two principal consequences. First, the partial removal of CENP-C and the KMN network components from mitotic centromeres. This removal requires microtubules and is dynin-dependent. Consequently knockdown of the dynin heavy chain or the ZW10 protein greatly rescues the Falafel depletion phenotype. Secondly, the PPP4 knockdown results in a defective spindle assembly checkpoint, manifested by the lack of arrest after colchicine treatment. This phenotype is microtubule-independent and therefore it may involve a mechanism different from the mitotic misplacement of CENP-C and the KMN network components. We are currently searching for the PPP4 substrate(s) having kinetochore function, whose continued phosphorylation could account for the PPP4 depletion-induced phenotypes.

**SW02.S6–16**

**Siroheme as an intermediate in the biogenesis of heme and heme d1; a new branch of tetrapyrrole synthesis**

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A new branch of the modified tetrapyrrole synthesis is described, revealing how both heme and heme d1 can be synthesised from siroheme. For heme d1 synthesis siroheme is decarboxylated to give dicarboxysiroheme, in a reaction catalysed by NirD-L, G and H. This newly described intermediate is then likely acted upon by NirJ to remove two propionate side chains and help generate the final d1 product. For the alternative heme biosynth-
sis (Abb) pathway it is shown that homologues of NirD and H, AbhA and B, also convert siroheme into diidcarboxylosiroheme. The latter is then acted upon by two radical SAM enzymes, AbhC and D, to oversee its transformation into heme via Fe-co-

corporphyrin III. The evolutionary relationship of these pathways to the various prosthetic groups is discussed.

SW02.S6–17
Mutation to alter the substrate specificity of a thermophilic L-rhamnose isomerase from Thermoanaerobacterium saccharolyticum NTOU1
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L-rhamnose isomerase (EC 5.3.1.14, L-RhI) mainly catalyzes the reversible aldose-ketose isomerization between L-rhamnose and L-rhamnulose. L-RhIs can also catalyze the conversions between other aldoses and their corresponding ketoses, such as L-mannose and L-fructose, L-lyxose and L-xylulose, D-ribose and D-ribulose, and D-allose and D-psicose. This capability has the potential to produce various rare sugars that are monosaccharides and rarely distributed in nature, such as D-allose. D-Allose has been shown to be able to suppress the growth of Cu9-22 and DU145 cancer cell lines, and to confer rice plant resistance to the rice pathogen Xanthomonas oryzae pv. oryzae. Due to the poor substrate specificity of T. saccharolyticum NTOU1 L-Rh against D-allose, mutations L59A, L59E, L59F, L59M, I102A, I102E, and I102Q located near substrate binding area were constructed to reduce the Km values toward D-allose. The catalytic efficiencies (kcat/Km) of L9A, L59E, L59F, L59M, I102A, I102E, and I102Q L-RhIs toward D-allose are 32, 50, 25, 54, 79, 121, and 139% respectively, of that of wild-type enzyme. The increased catalytic efficiency of I102Q L-RhI against D-allose suggests that this mutant enzyme has the potential to be applied in the process of rare sugar production.

SW02.S6–18
X-ray study of molecular oxygen reduction by fungal laccase from basidiomycete Steccherinum murashkinskyi
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Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) represent the largest subgroup of blue multicopper oxidases catalyzing the oxidation of a broad range of substrates with the four-electron reduction of dioxygen to water. Active site of laccases contains one type 1 copper (T1), one type 2 copper (T2) and two type 3 copper (T3) ions. Moreover, T3 and T2 form a tri-nuclear cluster performing a reduction of molecular oxygen. In spite of the fact that a lot of laccase catalysis studies have been performed over the last 20 years, the mechanism of oxygen reduction to water still remains unclear, although some of its aspects have been unraveled with the help of X-ray crystallography.

The aim of the present work was to study the catalytic mechanism of fungal laccase monitoring the structural changes in the T2/T3 cluster occurred under elevated doses of X-ray radiation. The easy crystallization of laccase from basidiomycete Steccherinum murashkinskyi allowed us to carry out these X-ray experiments. The single Lac crystal was used for collecting the 16 data sets during 8.5 h. As a result 16 structures of laccases intermediates have been solved with resolution 1.34–1.5 A. The electronic density of 15 data sets can be described as mixture of two forms of T2/T3 tri-nuclear cluster enzyme: oxidized (with water ligand inside cluster) and reduced (oxygen ligand inside cluster). And the 16-th data set was described as completely reduced enzyme. Thus, the process of oxygen reduction under X-ray was documented and the T2/T3 cluster structures in both oxidized and reduced forms were established. The mechanism of molecular oxygen reduction by laccase from Steccherinum murashkinskyi has been proposed based on the structural data obtained.

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SW02.S6–19
Exploration of the function of human methyltransferase like 23 (METTL23)
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Methyltransferases (MTases) are enzymes affecting a wide range of biological processes in all organisms encompassing signal transduction, gene regulation, protein and DNA repair, biosynthesis, chromatin regulation and gene silencing. In humans, many methyltransferases are linked to diseases such as cancer, inflammation, and neurodegeneration. The majority of methyltransferases are S-adenosyl-l-methionine (SAM) methyltransferases which catalyze the transfer of a methyl (CH3) group of SAM to various substrates such as nucleic acids, proteins, lipids, or small biomolecules. Generally, the enzymes share a reaction mechanism where the nucleophilic acceptor attacks the electrophilic carbon of SAM via an SN2 displacement yielding a methylated biomolecule and S-adenosyl-l-homocysteine (SAH) as products. The human genome contains many uncharacterized methyltransferases. Recently, a putative methyltransferase was identified on the human chromosome 17 (termed methyltransferase-like 23, METTL23). Based on amino acid sequence similarity, METTL23 is a member of the methyltransferase superfamily and was classified as a SAM-dependent methyltransferase. In this study, we attempt to express METTL23 in several host systems such as Escherichia coli and Pichia pastoris, to generate protein for biochemical and structural studies. Following protein expression and purification, we will investigate its catalytic properties with regard to substrate preference and kinetic parameters. Eventually, it is our goal to determine the three-dimensional structure and develop a better understanding of its biochemical and physiological role in humans.

SW02.S6–20
EDC4 interacts with and regulates the dephospho-CoA kinase activity of CoA synthase
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Coenzyme A synthase (CoA Synth) is a bifunctional enzyme which facilitates the last two steps of Coenzyme A biogenesis in higher
SW02.S6–21
Inhibition kinetics of sheep brain cortex glucose 6-phosphate dehydrogenase by metal ions
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Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP+/NADPH oxidoreductase, EC 1.1.1.49) (G6PD) functions as the first rate-limiting enzyme in the pentose phosphate pathway, responsible for the generation of NADPH in a reaction coupled to the de novo product ion of cellular ribose. G6PD deficiency is a common enzymatic defect. Deficiency of the enzyme leads to haemolyis in the presence of oxidative stress. G6PD has been purified from sheep brain cortex cytosol. The purification procedures included high-speed ultracentrifugation, 2°, 5°-ADP-Sepharose 4B affinity chromatography and DEAE Sepharose Fast Flow anion exchange chromatography. Purity control of the enzyme was performed with SDS polyacrylamide slab gel electrophoresis and Sepharose G-200 gel filtration chromatography. The enzyme was obtained with a yield of 68.33% and had a specific activity of 51.25 U/mg protein. The overall purification was approximately 9440-fold. In this study we have tested some of the essential transition metal ions on the G6PD enzyme such as zinc (Zn²⁺), nickel (Ni²⁺), copper (Cu²⁺) nonessential metals cadmium (Cd²⁺), lead (Pb), mercury (Hg²⁺). Assays of G6PD in the presence of heavy metal ions were performed in the system without enzyme-inhibitor preincubation, in which the reactions are initiated by adding enzyme to a substrate-inhibitor mixture (0.005–4 mM inhibitor concentrations). Kinetic characterization of the inhibition is also investigated. Ki is calculated from Statistica program. Zinc, copper, nickel inhibition is competitive with respect to both (G6P) and (NADP⁺) KM GAP0 0.060 ± 0.02 mM, Ki 0.301 ± 0.076 and IC50 is 0.030 mM. Copper inhibition is Ki 0.235 ± 0.012 mM, Ki 0.773 ± 0.009 and the obtained IC50 value of copper is 0.075 mM. Nickel inhibition is Ki 0.177 ± 0.010 mM and Ki 0.168 ± 0.008 mM and obtained IC50 value of nickel is 0.025 mM. Cadmium inhibition is noncompetitive Ki 0.024 ± 0.012 mM and Ki 0.031 ± 0.002 mM and obtained IC50 value of cadmium is 2.0 mM. Mercury Ki 0.22 ± 0.19 mM and Ki 0.034 ± 0.007 mM and the obtained IC50 value of cadmium is 0.35 mM.

SW02.S6–22
Mechanistic and structural study of BcGT
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A marcolride glucosyltransferase (BcGT) from Bacillus cereus was overexpressed and purified. The enzyme catalyzes the reaction of transferring glucosyl moiety from UDP-Glu to various acceptors to form beta-configuration of O-glycosidic products. The enzyme exhibits a broad specificity on glucosyl acceptor, whereas the UDP-Glu is strictly required. Many flavonoids such as kaempferol, quercetin, apigenin, genistein, naringenin and luteolin have been tested as the glucosyl acceptor with good yield. Among them, quercetin is an interesting target. It is a common constituent in the diet and has been claimed to exert beneficial health effects. In addition to its anti-inflammatory and antioxidant activities, quercetin also exerts cardiovascular disease prevention effect and anti-cancer effect. The nature of glycosylation is known to influence the efficiency of quercetin absorption and biological function. In this study we demonstrate site-directed mutagenesis on acceptor binding site. Mutation of Phe240 drastically changed the regioselectivity of quercetin glycosylation and catalytic activity. These mutants become tremendously useful for enzymatic preparation of various types of quercetin glucosides.

SW02.S6–23
Substrate specificity and subsites role of a recombinant digestive cathepsin L-like proteinase of Tenebrio molitor
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Cathepsin L, a cysteine proteinase, is the major digestive proteinase in the beetle Tenebrio molitor. Previous studies of our group showed that there is one lysosomal (CAL1) and two digestive (CAL2 and CAL3) cathepsins L in its midgut. The 3D structures of the digestive enzymes were elucidated and we aim to study in details their specificities. CAL3 was expressed as a zymogen, purified and activated. Activity assays were performed with 57 fluorescent resonance energy transfer (FRET) peptides derived from the lead sequence Abz-klRSKQ-EDDnp. The parameters kcat and Kd were used in the determination of the substrate hydrophobicity (H) and the different subsites roles in catalysis and/or substrate binding by the ratio (n) of the thermodynamic parameters total activation energy (ΔG‡) and free energy of binding (ΔG). According to Marana et al. 2002, subsites that present n > 1 are involved primarily in catalysis, while an n < 1 suggests that they favor substrate binding. The data obtained suggest that the S1 subsite is hydrophilic (H = –2.4) and is involved in substrate binding (n = 0.6 ± 0.2). Its 3D structure shows that this subsite is located on the surface of the enzyme and is composed by different types of amino acids. This supports the experimental observation that this subsite accepts amino acids with small or bulky side chains in P1, with hydrophobic, polar or charged side chains. The S1′ subsite is also hydrophilic (H = –0.4), has a role in catalysis (n = 1.2 ± 0.2) and has no remarkable selectivity for any amino acid at the P′1 position. The 3D structure shows that this subsite contains part of the oxyanion hole, which stabilizes the tetrahedral intermediate, and it supports the experimental findings. The S2 subsite is hydrophobic (H = 1.3), which is in agreement with the available data for cathepsin L, and is involved in catalysis (n = 1.5 ± 0.2). This subsite in buried in the enzyme structure and its pocket is composed mainly by hydrophobic amino acids. Its role in catalysis may be promoted by a lowering of the intermediates chain tension. This study will be extended to S2′ and maybe to S3′ in order to identify the specificity in the substrate unprimed side and a molecular docking of the enzyme and some substrates will be attempted.
**SW02.S6–24**

**Two forms of laccase from fungus *Cerrena unicolor*: preparation, properties and crystallization**

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Laccase is an enzyme catalyzing oxidation of different compounds by oxygen that reduces to water during the reaction. It is widely spread among different groups of organisms but the most active producers of laccases are fungi. Laccase belongs to a multicycopper oxidases family of enzymes and contains four copper ions in its active center. Laccase is a suitable instrument of biotechnology and can be used for delignification and bleaching of plant fibers, detoxification and decoloration of industrial waste dyes, in organic synthesis, in cosmetics and medicine.

A fungus *Cerrena unicolor* VKM F-3196 produced two isoforms of laccase (LacC1 and LacC2) under growing in media with Cu²⁺ as inductor. Both isoforms catalyzed oxidation of different phenolic compounds. Analysis showed that LacC1 and LacC2 were products of two different genes, but they shares 97% identity of amino acid sequences. Meanwhile these isoforms had the same pH optima of ABTS oxidation (below 2.7) and distinct pH optima of 2,6-dimethoxyphenol oxidation (LacC1 – 4.4; LacC2 – 3.8). The isoform LacC1 was more stable at 70°C and retained more than 40% of its activity within an hour, while LacC2 activity is almost completely lost in the same period. Molecular mass of LacC1 was 75 kDa and of LacC2 was 67 kDa. The degree of glycosylation of LacC1 was 16% and of LacC2 was 6%. After removal of the sugars from the molecule of laccase both isoforms had the same molecular mass – 63 kDa.

We crystallized LacC2 by the hanging-drop vapour-diffusion method using PEG 6000 as crystallizing agent, and preliminary X-ray crystallographic is ongoing.

**SW02.S6–25**

**Cellular ATP of iron- and sulfur-oxidizing bacteria as an indicator of cell energetics and growth**

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To monitor growth and cellular ATP content of *Acidithiobacillus ferrooxidans* growing with ferrous iron and elemental sulfur, a bioluminescent assay was applied. The inhibitory effects of acidity and inorganic substrates on luciferase were reduced to negligible levels by diluting the culture samples. A linear relationship between the cellular ATP and the rates of cell growth and substrate oxidation up to the stationary phase was observed. The maximum ATP levels in *A. ferrooxidans* grown with Fe²⁺ and S⁰ were 1.16 and 0.33 amol per cell, respectively. The low value for sulfur-oxidizing bacteria was attributed to reduced sulfur bioaccessibility resulting in limitation of sulfur-oxidizing activity by the sulfur substrate. In response to the addition of ferrous iron at the stationary phase, which was caused by depletion of ferrous iron, the rates of growth and iron oxidation increased to the original levels. The intensity of bioluminescence was in agreement with these changes. Contrary to the iron-oxidizing culture, the ATP content of sulfur-grown cells decreased anomalously due to a culture pH increase at the stationary phase, which was caused by sulfuric acid inhibition instead of sulfur limitation. Although the rates of growth and sulfur oxidation reached the original levels, the ATP content of the culture remained constant because of gradual decrease in the cellular ATP. This different cellular metabolic mode resulted in limitation of the bioluminescence method use to monitor biomass growth, which should also be considered for other organisms before application of the ATP kit. The results defined conditions under which biomass growth and cell energetics could be monitored by the ATP assay to study biogeochemical activities of acidophilic iron- and sulfur-oxidizing bacteria.

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**SW02.S6–26**

**Production of cyclodextrins using purified cyclodextrin glycosyltransferase from *Thermoanaerobacter* sp. P4**

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Cyclodextrin glycosyltransferase (CGTase; E.C.2.4.1.19) is a member of amylase family (Family 13) of enzymes which produces cyclodextrins (CDs) from starch and related substrates by cyclization reactions. CDs have torus like shape with an hydrophilic outer surface and hydrophobic cavity. This unique shape allows them to trap variety of hydrophobic compounds by forming inclusion complexes, thus making them soluble. CDs are widely used in industries such as, pharmaceutical, textile and food. In this research, effect of substrate concentration, enzyme dose and temperature on CD production from purified thermo-stable CGTase of *Thermoanaerobacter* sp. P4 was investigated using potato starch as substrate. Enzymatic reactions were carried out without prehydrolysis and gelatinization of starch, i.e. enzyme was directly added to the starch solutions. Effect of substrate concentration was tested using 0.1 U/g starch and maximum yield (34.2%) was obtained with 4% starch. Substrate inhibition has been observed at higher concentrations and yield decreased to 8.8%, when 20% starch was used. 0.1–4.0 U enzyme per g of starch was used in order to determine the effect of enzyme dose on the production and maximum CD yield (38.4%) was obtained when the enzyme dose was 0.5 U/g starch. Effect of temperature was tested at temperatures ranging from 65 to 95°C and optimum temperature was 80°C. These results indicated that due to its thermostability, CGTase from *Thermoanaerobacter* sp. P4 can produce CDs in one step. Thus gelatinization and hydrolysis of starch can be eliminated that offers advantage for industrial use.

**SW02.S6–27**

**Interplay between the trigger loop and the F loop in the active centre of bacterial RNA polymerase during catalysis**

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Bacterial RNA polymerase (RNAP) is a complex molecular machine that performs highly processive RNA synthesis and is also able to catalyze RNA cleavage to remove incorrectly incor-
porated nucleotides. The key role in RNAP catalysis is played by the trigger loop, a flexible RNAP element that encloses nucleotide substrates in the active centre during RNA synthesis and is also involved in RNA cleavage. We recently proposed that the F loop located near the trigger loop contributes to the catalysis of nucleotide addition by RNAP by affecting structural changes of the trigger loop. To provide direct evidence in support of this hypothesis, we analyzed mutations in the trigger loop and the F loop in the active centre of *Thermus aquaticus* RNAP. We found that mutations in the F loop dramatically affected RNA synthesis only if the intact trigger loop was present in the active centre. Furthermore, we showed that substitutions and deletions in the F loop significantly changed the rate of RNA cleavage by RNAP. Analysis of the activity of mutant RNAP variants at various temperatures suggested that the F loop promotes temperature-dependent trigger loop transitions and plays an adaptive role in catalysis by RNAPs from thermophilic and mesophilic bacteria. Thus, the F loop modulates structural dynamics of the RNAP active centre during catalysis and may play a role in RNAP regulation by various factors.

**SW02.S6–28**

**Hydrolitic activity of adenosinetriphosphatases (ATPases) measured by a new experimental method**

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The detection of small amounts (nanomoles) of inorganic phosphate has a great interest in biochemistry. In particular, phosphate detection is useful to evaluate the rate of hydrolysis of phosphatases, that are enzymes able to remove phosphate from their substrate by hydrolytic cleavage. The hydrolyzation rate is correlated to enzyme activity, an extremely important functional parameter.

Cation transporting adenosinetriphosphatases (ATPases) are phosphatases that produce inorganic phosphate by cleavage of the gamma-phosphate of ATP [1]. These membrane transporters have many physiological roles and are emerging as potential drug targets. ATPase hydrolytic activity is measured to test enzyme functionality, as well as to provide useful information on possible inhibitory effects of molecules that interfere with the hydrolytic process.

We have optimized a molybdenum-based protocol that makes use of potassium antimony (III) oxide tartrate to allow its use with phosphatase enzymes [2]. The method was successfully applied to native and recombinant ATPases to demonstrate its reliability, validity, sensitivity and versatility. Our method introduces significant improvements to well-established experimental assays, which are currently employed for ATPase activity measurements. Therefore, it may be valuable in biochemical and biomedical investigations of ATPase enzymes, in combination with more specific tests, as well as in high throughput drug screening.

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**References**


**SW02.S6–29**

**Biological function of the NudC Nudix protein from plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000**

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Nudix hydrolyses are widely distributed pyrophosphatases with a conserved amino acids motif GX3E5[XA][XRE2][EXGU]. They hydrolyze a variety of nucleoside diphosphate derivatives. The proposed role of Nudix enzymes is to maintain cellular homeostasis. Recently several reports have indicated that some of these proteins from pathogenic strains of *E. coli* K1, *Legionella pneumophila* and *Pasteurella multocida* may act as virulence factors since they were required for accurate invasion processes.

We have established *in vitro* that NudC protein acts as NADH hydrolase. We have found that genomic disruption of the *nudC* gene severely impairs morphology, growth and swimming, swarming and twitching motility of *Pseudomonas* cells. Moreover, over-expression of the NudC protein has lethal effect on *P. syringae* cells.

In order to establish whether the catalytic activity of NudC protein is essential for its biological function mutagenesis studies were conducted.

The catalytic region of Nudix enzymes comprises the conserved 23-residue amino acids sequence indicated above. It is well documented that this sequence constitute a functional module essential for hydrolysis of pyrophosphate bond in Nudix substrates and that the particular amino acids from this motif cannot be replaced without losing catalytic activity by the mutated protein. Using site-directed mutagenesis two conserved residues, Glu190 and Glu194 were substituted individually by Gin in the NudC protein, and the enzymatic activity of these mutants was analyzed *in vitro*. In addition, the impact of the overexpressed mutated proteins on *P. aeruginosa* viability was tested.

We have established that both mutated proteins are catalytically inactive and, contrary to the wild type NudC, while overexpressed have no effect on the bacterial cells viability. These results suggest that the enzymatic activity of NudC is crucial for its biological function. Taken above into account and given that the preferred substrate of this hydrolase is NADH, a component of NAD+/NADH redox couple, it could be suggested that NudC is a novel factor involved in regulation of cellular redox balance in the bacterial cell.

**SW02.S6–30**

**Differential phosphorylation of Akt isoforms by protein kinase CK2: biochemical evidences and functional implications**

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CK2 is a ubiquitous and constitutively active Ser/Thr protein kinase which phosphorylates many substrates and plays a global anti-apoptotic role. It is usually overexpressed in tumor cells and, although it cannot be considered a direct cause of cancer, it potentiates other survival pathways, thus significantly contributing to the amplification of tumorigenic signals. PI3K/Akt signaling is one of the pathways positively controlled by CK2 at different levels. Akt (also known as PKB) is a survival kinase frequently up-regulated in cancer; under normal conditions, it is activated by external stimuli which induce its phosphorylation at two crucial sites, Thr308 (by PDK1) and Ser473 (by mTORC2). Three
isofoms of Akt exist, and among them Akt1 and Akt2 are the most widely and highly expressed. They share the same structure and activation mechanism, and they have many overlapping functions with a general pro-survival action; however, also isoform-specific roles have been recently reported, which apparently depend on the cell type. We have previously found that CK2 can directly phosphorylate Akt1 at Ser129; this causes a hyperactivated state of Akt, partly due to its increased association to the chaperone protein Hsp90, and consequent protection of phospho-Thr308 from dephosphorylation. Akt2 has never been analyzed for its phosphorylation by CK2 at Ser131, the residue homologous to Ser129 of Akt1. Here we investigate the possibility that differential phosphorylation by CK2 of Akt isoforms 1 and 2 occurs in certain cell types, we analyze the biochemical mechanisms underlying the differences, and we evaluate if this phosphorylation might be related to the divergent cellular functions of Akt isoforms.

**SW02.S6–31**

**The structure of two ferryl-oxo intermediates at the same oxidation level in the heme-copper binuclear center of cytochrome c oxidase: the protein effect**

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Identification of the intermediates and determination of their structures in the reduction of dioxygen to water by cytochrome c oxidase (CcO) are particular important to understanding both O2-activation and proton pumping by the enzyme. In this work, we report the products of the rapid reaction of O2 with the mixed valence form (Cu3+2+, heme a3++, heme a3++, heme a3+++Cu3++) of the enzyme. The resonance Raman results show the formation of two ferryl-oxo species with characteristic Fe(IV)-O bond. In contrast to previous work, the H-bonded form of His411 determines the strength of the distal Fe(IV)=O bond. In contrast to previous proposals, the P4 intermediate is also formed in the reaction of F−. These results suggest that the fully reduced enzyme, the unique proton pumping Fe(IV)=O stretching modes at 790 and 804/cm at the peroxy oxidation level (PAO). Density functional theory calculations show that the protein environment of the proximal H-bonded His411 determines the strength of the distal Fe(IV)=O bond. In contrast to previous proposals, the P4 intermediate is also formed in the reaction of Y167F with O2. These results suggest that in the fully reduced enzyme, the unique proton pumping Fe(IV)=O stretching modes at 790 and 804/cm at the peroxy oxidation level (PAO). Density functional theory calculations show that the protein environment of the proximal H-bonded His411 determines the strength of the distal Fe(IV)=O bond. In contrast to previous proposals, the P4 intermediate is also formed in the reaction of Y167F with O2. These results suggest that in the fully reduced enzyme, the unique proton pumping Fe(IV)=O stretching modes at 790 and 804/cm at the peroxy oxidation level (PAO). 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ment for high copper ion concentrations (a potentially toxic redox-active metal). It known that to facilitate the uptake and delivery of adequate levels of copper, some methanotrophs such as Methylosinus trichosporium OB3b and Methylococcus capsulatus (Bath) possess a specialized copper trafficking mechanism that involves the use of a small, water soluble, chromopeptide called methanobactin.

In this study we represent investigation of expression of the methanobactin by Methylococcus capsulatus (M). It was established that to maximize methanobactin production bacteria were grown in copper-deficient nitrate minimal salts growth media (5 μM CuSO4). For each harvest, the spent medium was centrifuged at 6000 g for 30 min to pellet cells. The supernatant was filtered through 0.22 μm membrane filters (MFS-OS-1 (Vladipor) and methanobactin was isolated from the spent medium by resin extraction (Diaion HP-20, Supelco). As isolated methanobactin possessed an absorption maximum at 340 nm and at 360 nm, and somewhat featureless absorption in the 220–280 nm range. The methanobactin was investigated by electrospray ionization time-of-flight mass spectroscopy.

In this work we have produced for the first time methanobactin from Methylococcus capsulatus (M).

**SW02.S6–34**

Regioselectivity of alpha-galactosidase from Thermotoga maritima in hydrolysis and transglycosylation reaction. Impact of non-enzymatic mutarotaton process on the observed hydrolytic activity

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Glycoside hydrolases are known for their hydrolytic activity towards different types of glycosidic linkages and wide regioselectivity. Alpha-galactosidase from Thermotoga maritima (GH 36) is the retaining glycoside hydrolase according to reaction mechanism and therefore shows transglycosylation activity. In this aspect the basic study on regioselectivity of the enzyme established its natural selectivity towards 1–3 glycosidic linkage. The model method of kinetic parameters determination based on model step-by-step hydrolytic cleavage of labelled oligosaccharides revealed significant impact of non-enzymatic process into hydrolysis reaction. Mutorotation of alpha-galactose to the beta-form present in the reaction appears to be definitely considered in long and short-term reaction mechanism, since galactose is the competitive inhibitor.

**SW02.S6–35**

Molecular aspects of tissue-specific regulation of canonical and non-canonical functions of aminoacyl-tRNA synthetases using tryptophanyl-tRNA synthetase as the example

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Aminoacyl-tRNA synthetases (ARSase) catalyze the specific aminoacylation of tRNA and play a key role in ensuring the accuracy of translation of the genetic code. ARSase are an ancient enzymes, which, in addition to its basic canonical functions in the cell, developed in the course of evolution, a range of additional non-canonical regulatory activities. The universality of these regulatory activities in the fact that they have appeared and have been developed by another prokaryotic unicellular stage of life forms, in order to implement a series of protective adaptive responses formed microbial biofilms, called ‘quorum sensing’.

Regulatory non-canonical functions ARSase, because of their great importance, needs a variety of multi-level control and regulation, and the mechanisms, with the emergence of data on the important role of epigenetic protein-protein interactions are based on a variety of different protein factors and enzymes. In light of the discovery of new important noncanonical functions of ARSase and tryptophanyl-tRNA synthetase (TRSase), in particular, is very actual disclosure of tissue-specific mechanisms for the control and regulation of the switch from the primary to the non-canonical functions TRSases. We during the long-term studies to open and examine in detail not only the important non-canonical functions of TRSases consisting in synthesis Ap3A, but the role of an important TRSase cofactor – zinc (Zn²⁺) in the regulation of the process of switching functions of the enzyme. The optimum level of zinc in the tissues (particularly in the pancreas) provides a protective adaptive action, including determining the ratio of apoenzyme and active TRSase, and, accordingly, the level of a modified form of the enzyme, which has a strong anti-angiogenic activity.

**SW02.S6–36**

The role of tryptophanyl-tRNA synthetase in the regulation of protective reactions cascades and homeostasis at the tissue and organismal level

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It is now widely known that the complex of traits, defined as the metabolic syndrome (MS) dramatically increases the risk of developing cardiovascular disease. At the molecular level, the MS and its implications constitute overall balance disorders between inflammatory and anti-inflammatory pathway. Among the list of genes with abnormal features include peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 alpha), leading to reduced levels of oxidative phosphorylation in the tissue and, consequently, to an increase in triglycerides level. Central role in the cascade of the defensive response belongs to nitrous oxide (NO), which is a second messenger, and the genes that control NO synthesis in the endothelium (eNOS). Optimal concentrations of NO stimulate the activity of coactivator PGC-1 alpha, and, directly or indirectly, activate the endocrine function of adipocytes, increasing the stability and function of endothelial cells and vascular smooth muscle. At the same time, during the activation of mitochondrial biogenesis gene NRF2 and reactions cascade counterreacting oxidative stress in the endothelium are activated.

We in the course of the molecular structure-function studies of one enzyme of the aminoacyl-tRNA synthetase class – tryptophanyl-tRNA synthetase (TRSase) opened an additional activity in the form of synthesis diadenosine triphosphate (Ap3A). This compound has been found to have a large range of regulatory activities, including the functions of neurotransmitters, regulation of blood flow, the regulation of platelet function and blood flow in the development of myocardial ischemia. An important function Ap3A, in our opinion, is the ability to significantly stimulate the release of insulin from the B-cells of the pancreas. However, we believe the presence of the more important, the basic regula-
Dinucleoside polyphosphates (ApnA) have a large range of regulatory functions at different levels of organisms, ranging from the highest forms of multicellular till lower unicellular forms. The importance and universality of regulatory functions of the ApnA follows from the known role of this metabolite in the formation of microbial biofilms in the implementation of adaptive features named ‘quorum sensing’ in communities of microorganisms. Acting as a second messenger as extracellular signaling molecules, ApnA can function as neurotransmitter regulators of blood flow as a vasoconstrictor and vasodilator, depending on the length of the phosphate chain. ApA, ApA activate or inhibit the development and the ability to aggregate platelets, stimulate the biosynthesis of DNA in cells. Based on our findings and the literature data, is well established that one of the main sources of the ApnA in the body of higher organisms are aminoacyl-tRNA synthetases (ARSase), in particular, tryptophanyl-tRNA synthetase (TRSase). TRSase is involved in the synthesis of ApnA, but not ApnA, due to the presence of discovered by us significant for catalytic activity zinc ion in the enzyme. The presence of a wide range of regulatory functions of TRSase which is synthesized primarily as apoenzyme, requires specific, well-functioning regulatory mechanisms at the level of protein that can switch from the basic canonical to additional, non-canonical activity. High importance of the non-canonical regulatory activity of the ApnA synthesised by TRSase confirms the involvement of these metabolites in the startup stages of defense reactions in the body, in particular the synthesis of nitrous oxide (NO) in the tissue, which is one of the key regulators of homeostasis of the cardiovascular system.

The studies revealed a subtle mechanism of interactions of different types of covalent modification of the TRSase with substrates and intermediates of enzymatic reactions (Trp, PPI, ATP) and, in particular, with zinc for tissue-specific implementation of the non-canonical functions, including the controlled synthesis of an important secondary messenger of cells and tissues – ApnA.

**SW02.S6–37**

Dinucleoside polyphosphates as key regulators of biological processes in tissues and body: molecular aspects of their synthesis by aminoacyl-tRNA synthetases

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**SW02.S6–38**

Thermal stability and energy of deactivation of immobilized cell wall invertase in natural and synthetic hydrolgel polymers

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The greatest amount of invertase in the cell of yeast Saccharomyces cerevisiae is located in the cell wall. Enzyme immobilization offers technical and economic advantages. Industrial applications of immobilized biocatalysts have been gaining importance in recent decades as they are widely used. Hydrolgel materials are increasingly studied for application in biological sensing, drug delivery, tissues regeneration and food industry for number of reasons.

The aim of this work was comparison some of important kinetic parameters for enzyme reaction of a cell wall invertase immobilized in a few hydrolgel carriers. Enzyme was immobilized in different natural (alginate and gelatin) and synthetic polymers (polyacrylamide). Concentration of enzyme in all immobilized biocatalyst was the same. Differences in activity of obtained biocatalyst were observed, while no significant difference in pH and temperature optima, as well as in activation energy and Michaelis-Menten kinetics parameters were shown. Significant difference between these biocatalysts was observed in their thermal stability. Thermal stability of free and immobilized cell wall invertase was determined by monitoring the enzyme activity at a few different temperatures between 50 and 70°C. Deactivation constants (kd) were calculated from Arrhenius equation for all biocatalysts (free and immobilized) for various working temperatures. Obtained kd values are plotted in the form of Arrhenius plot, that is ln of kd against the inverse of absolute temperature, yielding the energy of deactivation, as the angular coefficient of the adjusted straight line, times the universal gas constant. The obtained values are 326, 592, 620 and 706 kJ/mol for free, polyacrylamide, alginate and gelatin immobilized cell wall invertase, respectively. Therefore, increase of the energy of deactivation of immobilized enzyme shown greater stability than free enzyme, but the greatest stability was proven for enzyme immobilized in gelatin hydrolgel.

**SW02.S6–39**

Immobilization of NAD+/NADH on magnetic nanoparticles and its selective oxidation and reduction reactions with mediated by galactitol- and lactate-dehydrogenases

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Redox reactions in biochemistry have an important function and they are catalyzed by dehydrogenases and oxidoreductases. These reactions have large application by bio fuels, sensing technology and biotransformation for the production of several interesting materials.

The oxidoreductases are working with co-factors NAD+/NADH and their recycling are essential for economical reason. The recycling and reusability is a big challenge in bio fuel, sensing and biotransformation technologies. Without recycling the use of oxidoreductases are more expensive than their products. Many attempts are made for the reusability these materials. The solution of this problem is in the same time the solution associated with hydride (hydrogen) generation. This study focus on the synthesis of NAD+/NADH functionalized Fe3O4 magnetic nanoparticles and their application on the redox enzyme reactions for developing a method which enable ease regeneration and reuse of cofactors.

First, silica coated magnetite particles were synthesized and 3-Aminophenylboronic acid (APBA) was attached on the surface of particles. Nicotinamide adenine dinucleotide (NAD) was immobilized on the magnetically responsive APBA attached magnetic support. X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and transmission electron micrograph (TEM) methods were used to characterize the surface modified magnetic nanoparticles.
NAD<sup>+</sup>/NADH functionalized Fe<sub>3</sub>O<sub>4</sub> nanoparticles were used for oxidoreductase mediated oxidation and reduction reactions. Galactitol dehydrogenase was used as catalyst. For oxidation reaction galactitol was used to obtain industrial important tagatose and for reduction, hydroxyacetone was converted into 1,2-propanediole. The recycling process was realized by using lactate dehydrogenase for the conversion of pruvate to lactic acid.

**SW02.S6–40**

Human flavin-containing monoxygenase 3 polymorphism and its effect on drug metabolism

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It is well recognized that patients administered a particular drug will exhibit significant inter-individual variability in their response to treatment. Currently, Personalized medicine which defines the specificity and dosage of drugs according to effectiveness and safety for each patient is a very fast growing area of research. Dosage strongly depends on the rate of metabolism that is primarily regulated by the activity of cytochromes P450 and flavin-containing monoxygenase 3 (hFMO3).

Human FMO3 is a microsomal enzyme capable of metabolising drugs containing a soft-nucleophile, usually sulphur or nitrogen. This enzyme has three common polymorphic variants, one of which is V257M (rs1736557). In this work, the wild type enzyme (WT) and the engineered V257M polymorphic variant were heterologously expressed in bacteria and purified with a yield of 12 mg/l of culture. The activity of the purified enzymes was subsequently measured towards several drug and/or drug candidates. Initially benzamidine, a nonsteroidal anti-inflammatory drug (NSAID) and a marker substrate of hFMO3, and sulindac sulphide, another NSAID used in the treatment of chronic inflammatory conditions, were tested. For benzamidine the results obtained with the purified enzymes showed no differences between the WT and the V257M polymorphic variant in the N-oxygenation of this drug. In the case of sulindac sulphide, the variant showed a decrease in its catalytic efficiency when compared to the WT enzyme. Subsequently two Aurora kinase inhibitors, Tozasertib (VX-680) and Danusertib (PHA-739358), which are anti-cancer drug candidates, were tested. The conversion of Tozasertib and Danusertib to their corresponding metabolites by the purified WT and V257M hFMO3 showed significant differences. In the case of Tozasertib, the V257M variant showed a catalytic efficiency, expressed as kcat/KM, similar to the WT (0.33–0.36 min/µM). On the other hand, in the case of Danusertib, V257M showed a 3.4 fold decrease in catalytic efficiency (kcat/KM = 0.05 versus 0.17/min/µM for V257M and WT, respectively). These data reveal how a simple V257M substitution ascribed to a single nucleotide polymorphism may affect drug metabolism and efficacy.

**SW02.S6–41**

Effect of Cathepsin L variant gen silencing over proliferation, viability and organization of mitotic spindle in cell lines of colorectal cancer (Caco-2) and uterine cervical cancer (HeLa)

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Cathepsin L is a cysteine protease often overexpressed in cancer cells. In our laboratory a new nuclear Cathepsin L variant has been described in HeLa and Caco-2 cells. This 60 kDa variant has a differential localization through the cell cycle, displaying a nuclear location in S phase whereas in mitosis it locates in the mitotic spindle where it colocalizes with alpha-tubulin. In the current work, we assess the effect of Cathepsin L variant gen silencing over proliferation, viability and organization of the mitotic spindle in Caco-2 and HeLa line Cells. For this purpose, siRNA duplex aimed at Cathepsine L variant was designed. Obtained results show that cell cultures transfected with siRNA show a partial decrease of Cathepsin L variant expression; its subcellular localization is altered with regard to its normal localization. On this matter, cells transfected with siRNA show a localization of Cathepsin L variant mainly in the cytoplasm, whereas control cells show mainly a nuclear localization. On the other hand, it is detected that Cathepsin L variant gen silencing significantly affects cellular proliferation, which is observed by means of the decrease of Bromodesoxiuridine incorporation (BrdU). Cellular viability analyzed by means of the MTT technique is also significantly affected; a decrease of viable cells or metabolically active cells is observed. Mitotic spindle organization in cells transfected with siRNA is seriously altered; a collapsed mitotic spindle is generated and alfa-tubuline localization is distributed in a disorderly way unlike control cells localization. These particular results agree with the ones obtained by means of the activity inhibition of Cathepsin L variant with specific inhibitors for C athеспin L. These results lead us to the conclusion that the presence of Cathepsin L variant would be relevant for cell survival and its overexpression would contribute to the tumoral phenotype given that cellular proliferation is encouraged.

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**SW02.S6–42**

Protective role of hyaluronic acid and hyaluronidase in the mechanism of overcoming carbohydrate deficiency shock by the culture of bacterial strain streptococcus zooepidemicus

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**Objective**: To study the molecular-genetic mechanism of overcoming the external physical effects on the microorganism of the unknown cellular stress factor in the form of carbohydrate deficiency shock (CDS) by the culture of bacterial strain ATCC 39920 Streptococcus zooepidemicus in a self-organizing system of cell-stress control.

**Methods**: Hyaluronic acid (HA) was determined using the carboxazole method or after exhaustive acidic hydrolysis by reacting N-acetylgalactosamine with the Elson-Morgan’s reagent. The total hyaluronidase activity was evaluated using viscometer. The measurements of cells and capsules, carbohydrate fermentation abil-
ity and other microbiological, biochemical, and physiological tests were performed according to standard procedures.

Results: As a result, the experimental and theoretical studies have provided evidence on the mechanism of manifestation of physical effects on cells of an unknown cellular stress factor in the form of CDS. This factor activates a kind of specific (to a given stress) closed-self-organizing system of cell control which remained ‘silent’ until the impact of stress. The purpose of this system is to reduce the effects of stress. In the control loop of the system one of the vital operons hyaluronate lyase operon hylA precursor is ‘built’. Initiation signal of transcription of the inducible gene operon is originated in the phosphotransferase system (PhTS), where the cell begins to undergo the impact of carbohydrate deficiency shock by means of a substantial reduction of the formation of phosphorylated glucose molecules. The subsequent processes of hylA precursor gene transcription and translation of its mRNA enable synthesis of the corresponding amino acid sequence of the inducible hyaluronate lyase protein. The synthesized enzyme serves to the main purpose of managing a self-organized system: the destruction of high-molecular hyaluronic acid to low molecular weight fragments and disaccharides, in order for the size of the latter toallow carrying out their active transport out of the PhTS through the pores of the cell membrane and implementing the process of fermentation of the available carbohydrates by the cell. This process allows maintaining normal levels of adenosine triphosphate and homeostasis of the cell until the exhaustion of HA in the culture liquid and on the capsules of bacterial strain.

Conclusions: The paper presents the evidence of the impact of the physical manifestation of an unknown cellular stress factor in the form of CDS on the cell, which was obtained in the study of the synthesis and degradation of HA problems using test strain. It is established that for protection from these and other stresses a long way of evolutionary adaptation has been taken by the cell to synthesize specific to a particular stress closed self-organized control system which goal is to reduce the impact of stress on the controlled object – a cell. In particular, for this culture of the strain the main goal is to reduce CDS effects. And this problem is effectively solved by the cell strain due to the molecular genetic defense mechanism to overcome CDS, incorporated in its control loop. The key role in this mechanism belongs to hyaluronic acid and bacterial hyaluronidase. The experimental part of the work with the test strain confirms the theoretical conclusions set above and allows us to formulate a systematic approach to the problem of cellular stress. The general concept of such system is as follows. First, the control system is ‘turned on’ by a specific external influence of habitat that induces cellular stress (thermal shock, CDS, etc.). Second, the system detects the signal and converts specific signal of stress into the initiation signal of inducible promoter for the gene of the operon according to the present cellular stress. Third, the synthesis of the protein of cellular stress is maintained by the system. Fourth, the role of this protein is to restore the ‘status quo’ of the cell. Fifth, when the normal vital functions of cell are restored, stress protein synthesis stops, and closed-loop control system is ‘turned off’ until the next manifestation of accident occurring cellular stress.

**SW02.S6–43**

**Molecular dynamics and QM/MM free energy profiles of cytosine C5-methyltransferase M.Hhai**

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The target of this work is the study of mechanism of the reaction catalyzed by M.Hhai, an enzyme that belongs to the restriction-modification system of the bacterium *Haemophilus haemolyticus* which catalyzes the methyl transfer from S-adenosil-l-metionine (SAM) to C5 position of a cytosine base of DNA, working at the 5′-GCGC-3′ sequence generating C5-methyl-cytosine.

The X-Ray structure [1] of the enzyme complexed with SAM and a DNA sequence was the starting point of our simulations. We performed all calculations considering the whole enzymatic and DNA environment and the solvation effect by including the enzyme in a orthorhombic box of TIP3P water molecules of $88 \times 87 \times 99$ Å of side, including sodium counterions to neutralize the charge of the system. Using the NAMD program we equilibrated the system by means of 10 ns of classical molecular dynamics (MD) with the AMBER force field, employing periodic boundary conditions, Ewald summations, a temperature of 300 K and a time step of 1 fs. We then performed 100 ns MD simulation in order to analyze the most important interactions formed between the enzyme and DNA, the interactions within the active site, how the unpaired base is stabilized and how the DNA helix accommodates the great perturbation that a flipped out base means for its structure [2].

To analyse the chemical reaction we performed 500 ps of quantum mechanics/molecular mechanics (QM/MM) MD simulation at 300 K using Dynamo program. Quantum subsystem was treated using the AM1 semiempirical hamiltonian adding corrections at the M062x/6-311+G level. By means of the on-the-fly string method [3] the minimum free energy path (MFEP) for each step of the reaction was obtained. Then, the path collective variable [4] was defined along these paths, to obtain the potential of mean force (PMF) using umbrella sampling.

**References**


**SW02.S6–44**

**Simulation tools for the automatic determination of enzymatic reaction mechanisms. The guanidinoacetate methyltransferase (GAMT) case**

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We present an approach for the semi-automatic determination of reaction mechanisms in condensed phases and particularly of enzymatic reactions. First, the on-the-fly string method [1] is applied to find the minimum free energy paths (MFEP) in a multidimensional space of several collective variables. This path is defined in terms of interatomic distances – a natural choice to study bond forming/breaking processes, although other coordinates, not only geometrical coordinates, can be easily imple-
mended. Then a reaction coordinate, a path collective variable or PCV, is defined along these paths to measure the advance of the chemical reaction [2]. By using this PCV the potential of mean force (PMF) is obtained, which facilitates the use of Transition State Theory to determine the rate constant of the process. This approach was applied to the study, by means of Quantum Mechanics/Molecular Mechanics simulations, of the reaction catalyzed by Guanidinoacetate Methyltransferase (GAMT). GAMT catalyzes the methylation of guanidinoacetate by S-adenosyl-L-methionine. This reaction involves a methyl transfer and a proton transfer with two possible reaction mechanisms proposed in the literature, differing essentially in the timing of the two transfers [3,4]. While a typical approach would require the determination of the Free Energy Surface along two coordinates (one for the methyl transfer and one for the proton transfer) our approach helped to distinguish which mechanism is the most probable one at a computational cost that is nearly 2 orders of magnitude lower.

References

SW02.S6–45
One-step purification and covalent immobilization of benzaldehyde lyase (BAL, EC 4.1.2.38) with chelate-epoxy modified magnetic solid support and its carbogiration reactivity
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Crude histidine-tagged recombinant benzaldehyde lyase (BAL, EC 4.1.2.38), a multimeric enzyme, was immobilized on the magnetically responsive epoxy-chelate magnetic support following a two-step mechanism; that is, the protein is physically adsorbed and subsequently, the covalent reaction takes place. With this two-step mechanism, the selectivity of metal chelate affinity chromatography and the covalent immobilization capacity of epoxy supports was combined in order to accomplish the purification, immobilization, and stabilization of a histidine-tagged recombinant benzaldehyde lyase. To fulfill this objective we prepared and characterized a multifunctional Co²⁺-IDA-epoxy functionalized Fe₃O₄@SiO₂ magnetic nanoparticles which are modified with glycidyloxypropyltrimethoxysilane (GPTMS) and iminodiacetic acid (IDA).

To test immobilized BAL, benzoin condensation reaction was performed with this magnetically responsive biocatalyst. The results obtained from the carbogiration reaction that was performed with this simple and convenient heterogeneous biocatalyst were comparable to that of free-enzyme-catalyzed reaction. Additional advantages is its reusability and it is easy to work with.

SW02.S6–46
The kinetics of binding of factor X to the activated platelet membrane
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Introduction: All major reactions of blood coagulation proceed on the negatively charged phospholipid membranes, which provide optimum interaction of the enzyme, cofactor, and substrate and increase the rate of reaction by several orders of magnitude. Predominantly binding of factor X (FX), a major coagulation zymogen, to the phosphatidylserine(PS)-positive subpopulation of platelets was shown earlier, but was poorly characterized. In addition, we recently discovered a new PS-positive subpopulation of platelets (Topalov et al. Arterioscler Thromb Vasc Biol 2012, 32:2475–2483). Here we studied interaction of factor X with all three subpopulations of activated platelets.

Methods: FX was covalently labeled with fluorescein. Platelets were activated at 2 × 108/ml with 100 nM thrombin in the presence of 2.5 mM CaCl₂ for 10 min. They were incubated with FITC-labeled FX at different time periods, diluted (if required), and immediately analyzed with an Accuri C6 cytometer. Fluorescence intensity was converted to a mean number of molecules per platelet using a calibration curve prepared with Green Flow Cytometry Intensity Calibration Kits.

Results: Three platelet subpopulations were detected upon activation. The two PS-positive subpopulations bound 50 000–65 000 molecules FX per platelet, with an apparent equilibrium Kd of 1340 ± 290 nM (r = 3). The PS-negative platelets bound 1000–2000 molecules FX per platelet, without clear saturation. Binding was calcium-dependent, reversible and specific as determined in the experiments in the presence of excess unlabeled factor. Prothrombin competed with FX for binding sites on the membrane. Unexpectedly, kinetics of dilution-induced FX dissociation from the PS-positive platelets revealed a multistep process, with a rapid (1–2 min) step followed by a plateau. As a result, the overall binding and dissociation process was a hysteresis-like process possessing a kind of ‘memory’.

Conclusions: Binding of FX to both PS-positive subpopulations is calcium-dependent, reversible, specific, but with a possibility of competition with prothrombin. Most importantly, this is a hysteresis-like process possessing a ‘memory’ that can be important for reactions in flowing blood.

SW02.S6–47
Evolution of a reactibody by combined natural and computational methods
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Immunoglobulins (Igs) play a crucial role in modern biopharma due to their unique ability to proceed the most adequate transfer of structural information to the required functionality. Sophisticated approaches aimed to generate highly evolved binding/catalytic properties of Ig molecule were developed dur-
ing last three decades. In vitro immunization became a very powerful tool allowing operate with large repertoires of Ig combining sites. However the lack of B cell maturation stage in those cases results in restricted functionality of Ig selected from the combinatorial libraries. Recently, the novel ‘reacti-body’ approach was developed based on the chemical selection of biocatalysts from immunoglobulin library followed by eukaryotic expression. The deep structural and functional analysis revealed architecture of active center with cholineesterase-like anion binding site, hydrophobic pocket and reactive Tyr residue. We propose to mimic Ig maturation with quantum mechanics (QM), molecular mechanic (MM) and molecular dynamic (MD) approaches to estimate optimal reacti-body structural insiders. This methodology is highly demanded when in vivo immunization is prohibited because of antigen toxicity or its instability in blood stream. We developed the computational maturation to provide best driving forces for ‘Ig paraxonase’. Theoretical evolution was combined with rational design and site-specific mutagenesis and probed by highly refined 3D X-ray data, pre-steady state kinetics and displayed high predictability.

SW02.S6–49
Biochemical characterization and classification of a novel metagenomic nicotinamidase
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Nicotinamidases catalyze the hydrolysis of nicotinamide to nicotinic acid and ammonia. In vivo, the latter compound is converted back to NAD⁺ in a series of reactions catalyzed by other enzymes in the NAD⁺ salvage pathways. Nicotinamidases are key enzymes in many organisms, including bacteria, mycobacteria, yeast, protozoa, plants and even invertebrates. However, no metagenomic nicotinamidases have been characterized. In order to search for new nicotinamidases, a metagenomic library from a subsurface mine microbial mat was screened, finding a positive clone, which was subcloned in pET28a and expressed in E. coli Rosetta 2 (DE3) at 1 mM IPTG and 25°C. The enzyme was pure after a two-steps purification procedure (HisTrap and gel filtration), having a molecular weight of 26 kDa in SDS-PAGE. The enzyme activity was both pH- and temperature-dependent, with maximal activity at pH 10.0 and 90°C, respectively. The enzyme showed a clear preference for nicotinamide (NAM) rather than pyrazinamide (PZA) with KM values of 0.11 and 0.33 mM, respectively. The catalytic efficiency for NAM was almost two-fold higher than for PZA. The enzyme was also modeled to design the mutational analysis towards five critical amino acids. The kinetic parameter and specificity for such mutants was also characterized. In addition, a phylogenetic analysis was carried out, finding the enzyme closed related to Acidobacteria nicotinamidases/pyrazinamidases.

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SW02.S6–50
Expression, purification and characterization of a novel NADH-dependent glutamate dehydrogenase from Geobacillus kaustophilus HTA426
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Glutamate dehydrogenases (GDHs) are ubiquitous enzymes that occupy an important branch point between carbon and nitrogen metabolism. They are involved in the reversible interconversion of t-glutamate to α-ketoglutarate and ammonia, using either NAD(H) or NAD(P)H as coenzyme. In this work, a NADH-dependent glutamate dehydrogenase was search to be used in high-throughput screening strategy for nicotinamide-hydrolizing enzymes based on a coupled enzymatic assay. A Blast search
revealed a putative NADH-dependent GDH in Geobacillus kau-stophilus HTA426 (GkGDH). This enzyme was cloned in pET28a and expressed in E. coli Rosetta 2 (DE3) at 0.8 mM IPTG and 30°C. The multimeric enzyme was purified after 100 kDa ultrafiltration, followed by ion-exchange and IMAC chromatography to yield a single 47 kDa band in SDS-PAGE. The enzyme activity was both pH- and temperature-dependent, with a maximal activity at pH 7.5–8.0 and at temperature around 65–75°C. The enzyme showed and strict dependence on NADH, no activity was found towards NADPH. GkGDH showed substrate inhibition for 2-ketoglutarate with a Km of 7.18 mM and a Km of 2.34 mM. The Km for NADH and NH4Cl were 46 μM and 33 mM, respectively. The enzyme was also modelled and an structural phylogenetic analysis was carried out, finding it in a separate primitive clade. Finally, the enzyme was used to find new evolved nicotinamidases from a directed evolution library.

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SW02.S6–52
Immobilization and characterization of trametes versicolor lacasse in porous silica particles
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In this work, several technologies has been used to produce siliceous materials, as spherical silica particles by sol-gel method with a mean size of 1 μm, porous silica nanoparticles with a diameter around 7 nm, and spherical siliceous meso cellular foam particles with a size mean of 5 μm, pores around 27 nm and narrow windows to link the pores of 15 nm. Also we have purchase commercial silica particles with mean sizes of 63, 75 and 200 μm. These materials have been employed as suitable carriers for laccase immobilization, for immobilization purposes the materials have been functionalized with reactive groups to couple the enzyme on the surface or in the pores of the silica particle. Firstly, the enzyme was adsorbed by charge difference due amine groups, and then the enzyme was anchored to the support with oxirane groups. It has been found that without the presence of amino groups, the adsorption on the support is very low, since more than 93% of the enzyme remained in the supernatant, with amino groups only 22% of the enzyme remained in the supernatant, and the best pH for the immobilization was around 7. The prepared materials showed good storage stability in phosphate buffer, and degradation was not observed. It has been observed that in porous materials the amount of absorbed enzyme was greater than the amount immobilized on solid supports and the enzyme activity recovered was also good for all the supports. To test the strength of binding of the enzyme to the support, we subjected one of the examples to several centrifugation cycles, and it was found no enzyme leaking from the support and no loss in the activity.

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SW02.S6–53
Enzyme bioprospecting for lignin valorization: searching for novel bacterial oxidases and peroxidases
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The oxidative enzymatic breakdown of lignin represents a huge potential towards the sustainable production of high value-added aromatic intermediates, currently being produced from petroleum based processes. Within this framework, significant research efforts have been devoted, over the last decades, in the discovery and exploitation of microbial enzymes involved in the degradation and/or modification of lignin. The enzymology of lignin modification is mostly studied in white- and brown-rot fungi and is much less understood in bacteria. However, recent findings indicate that within this kingdom, there are representatives with significant and novel ligninolytic potential.

In a bacterial enzyme bioprospecting effort for lignin valorisation, fifty bacterial strains isolated from a polluted field near an oil-refinery plant (Elefsina-Greece) were studied on their ability to grow on different aromatic substrates, including lignin derivatives, as carbon and energy sources. The majority of the isolates showed a distinct ability to grow on aromatic carbon sources especially caffeic acid, ferulic acid, catechol, quercetin and chlorophenol. 16s rRNA gene analysis revealed a significant phylogenetic diversity, with the most prominent strains belonging to the Bacillus and Enterobacter genera.

Two novel isolates, a Bacillus sp. and an Enterobacter sp. that revealed the greatest ability to grow in the above mentioned sources, where chosen for further study. Liquid cultures on different carbon sources, namely glucose, caffeic acid, catechol, tannic acid, veratryl alcohol and quercetin were conducted, in order to assess the corresponding extracellular and cell-associated enzymatic potential. Using high throughput microplate approaches, we performed an extensive screening for oxidase and peroxidase activities employing various substrates at different pH values, assessing in parallel the possible $H_2O_2$, Mn$^{2+}$ and NAD/H dependencies. The results suggest that both strains are able to produce lignin modifying activities in a manner reliant to the growth carbon source, with Enterobacter sp. expressing these activities on a wider carbon source spectrum compared to Bacillus sp. The measured lignin related activities were successfully correlated with the results of a bioinformatic analysis for possible genes responsible for the oxidative and/or hydrolytic activity on aromatic substrates within the Bacillus and Enterobacter taxa.

In overall, our results indicate the potential of these microorganisms as aromatic degraders and their possible exploitation as a source for novel enzymes for lignin valorisation.

SW02.S6–54
Solubility and lipophilicity of boron cluster pharmacophores
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Boron cluster moieties have been identified as promising pharmacophores. In addition to their application as high boron-content agents for boron neutron capture therapy and boron neutron capture synovectomy or as radioimaging or magnetic resonance imaging probes, boron cluster moieties can serve as constituents of enzyme inhibitors. For example, boron cluster containing compounds have been shown to inhibit HIV-1 protease, cyclooxygenase, serine protease, protein kinase C, and to modulate activity of nitric oxide synthases.

The pharmaceutical use of such compounds is, however, complicated by their low solubility and tendency to self-assemble in aqueous solution. We showed that human serum albumin (HSA) and several biocompatible excipients can effectively increase the solubility of boron clusters and suppress their self-assembly via non-specific complexation. HSA is a well-known drug carrier that is able to bind negatively charged and (hetero) aromatic compounds and transfer them to target tissues. HSA can solubilize poorly soluble drugs in the circulatory system via its ligand-binding ability and can also delay the metabolic clearance of therapeutic agents. From this point of view, the interaction of boron clusters with HSA is expected to play a key role in the potential use of boron cluster derivatives as drugs. We estimated the solubility of a vast series of boron clusters [mainly cobalt bis(dicarbollide) derivatives] in pure water, saline, and saline with HSA as a model of blood plasma. In addition, we determined the octanol-water partition coefficients (Pow) as a lipophilicity descriptor. Pow weakly correlates with the water solubility of boron clusters, whereas the ability of HSA to increase the solubility of boron clusters correlates well with their Pow values. Because boron clusters are known inhibitors of HIV protease, the possible correlation between Pow and ability to inhibit HIV protease was investigated. Results from this study indicate that interaction of boron cluster inhibitors with HIV protease is driven by specific binding rather than by promiscuous lipophilic interactions. The most promising candidates for further drug development were identified by ligand lipophilicity efficiency analysis.

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SW02.S6–55
Proteinase-binding loop does not significantly contribute to the specificity of recognition of serine protease factor Xlla by its canonical inhibitor
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Canonical serine protease inhibitors usually interact with their cognate enzymes through protease-binding loop. Here, we examined whether the protease-binding loop alone is sufficient to inhibit a serine protease or other parts of a canonical inhibitor are also involved. Activated coagulation factor X (FXa) and corn Hageman factor inhibitor (CHFI) were selected as a model system. FXIIa is a serine peptidase. CHFI is a trypsin inhibitor from corn that is also found to selectively inhibit FXIIa with a $K_i = 24$ nM [1]. CHFI has a canonical binding loop with a scissile bond between Arg-34 and Leu-35. Three Pro residues located closely to the cleavage site seem to decrease the chain flexibility.
SW02.S7 Protein Structure and Folding (II-S7)

SW02.S7–1 Insights into protein folding and evolution from the structural classification of proteins

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In the past two decades the complete genome sequencing of many hundreds of organisms has transformed our knowledge of protein sequences and their families. At the same time the progress of structural biology and structural genomics resulted in a dramatic increase of the number of known protein structures. Over the years we have been systematically classifying and exploring structural and probable evolutionary relationships amongst proteins of known structure, discovering many new relationships in the process. These include new types of relationships, which have potentially important implications for our understanding of the folding and evolution of protein structure. The examples of these non-trivial relationships are not many, but they grow in numbers as the protein data do.

SW02.S7–2 Breaking the amyloidogenicity code: bioinformatics approach to predict predisposition to amyloidosis

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Devastating neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s Disease, and Huntington’s disease are linked to the formation of protein aggregates called amyloid fibrils. There currently exists no cure, and no means of early diagnosis for these diseases.

Numerous studies have shown that the ability to form amyloid fibrils is an inherent property of the polypeptide chain. This has lead to the development of a number of computational approaches to predict amyloidogenicity by amino acid sequences. Although these methods perform well against short peptides (~6 residues), they generate an unsatisfactory high number of false positives when tested against longer sequences of the disease-related peptides and proteins [1].

Recently new experimental techniques have shed light on the structure of amyloids showing that the core element of many disease-related amyloid fibrils is a β-strand-loop-β-strand motif called β-arch [2–5]. Using this information we have developed a new approach to predict amyloidosis based on protein sequence analysis. Test of this program has shown that it yields the best known prediction of the disease-related sequences. As whole genome sequencing becomes cheaper, our method provides opportunity to create individual risk profiles for the neurodegenerative, age-related and other diseases ushering in an era of personalized medicine.

References

SW02.S7–3 Restrictions superimposed on protein folding by its size

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The ability of protein chains to spontaneously form their spatial structures is a long-standing puzzle in molecular biology. Experimentally measured rates of spontaneous folding of single-domain globular proteins range from microseconds to hours: the difference (11 orders of magnitude!) is akin to the difference between the life span of a mosquito and the age of the Universe. We show that physical theory with biological constraints outlines a ‘golden triangle’ limiting the possible range of folding rates for single-domain globular proteins of various size and stability, and that the experimentally measured folding rates fall within this narrow triangle built without any adjustable parameters, filling it almost completely. In addition, the ‘golden triangle’ predicts the maximal allowed size of the ‘foldable’ protein domains, and the size of domains found in known protein structures is in a good agreement with this limit.

SW02.S7–4 Unfolding of Torpedo californica acetylcholinesterase: effects of chemical and pharmacological chaperones

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The dimeric form of Torpedo californica AChE (TcAChE) provides a valuable experimental system for studying transition between native (N), partially unfolded and unfolded (U) states of a protein, since long-lived partially unfolded quasi-native (N*) and molten globule (MG) states can be generated by chemical
modification of Cys231, by chemical or thermal denaturation, and by oxidative stress. We have shown that the rate of unfolding at 28°C is greatly enhanced in the presence of liposomes. Arrhenius plots and differential scanning calorimetry reveal that in the presence of the liposomes the energy barrier for transition from the N to the MG state is lowered from 120 to 47 kcal/mol. Reversible cholinesterase inhibitors, such as tacrine, methylene blue and BW28c51, protect TeAChE against the destabilizing effect of the liposome surface as well as against thermal denaturation. Chemical modification Cys231 by the natural thiosulfinate, allicin, or by organomercurials, transforms TeAChE to a quasienative (N*) state. The modified enzyme is, however, metastable, and is converted spontaneously and irreversibly, at room temperature, with $t_{1/2}$ =100 min, to an MG state. Certain osmolytes, which act as chemical chaperones at molar concentrations, including trimethylamine-N-oxide, glycerol, and sucrose, as well as divalent cations, such as Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$, can prevent this transition of the N* to the MG state for $>$24 h at room temperature. The stabilizing effects of the osmolytes can be explained by their differential interaction with the native and quasienative states, resulting in a shift of equilibrium toward the native state. The divalent cations, as well as small reversible inhibitors of TeAChE, which can be considered as pharmacological chaperones, protect the native enzyme from unfolding by binding to specific sites (X-ray data), resulting in an increased energy barrier for irreversible transition to the MG state.

$$N \leftrightarrow N^* \leftrightarrow MG \leftrightarrow U$$

**SW02.S7–5**

**Structure and oligomerization of frataxin: insights into the mechanisms of iron delivery and detoxification in mitochondria**

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Frataxin a mitochondrial protein, which has been linked to the neurodegenerative disease Friedreich’s ataxia, is known to perform key functions in iron storage, delivery and detoxification in mitochondria. Using X-ray crystallography, single-particle electron microscopy and small-angle X-ray scattering (SAXS), we have studied the structures of the iron-free and iron-loaded frataxin trimers and 24-mers as well as the process of metal-dependent assembly of larger oligomers of the protein and compared oligomerization induced by the non-physiological metal Co$^{2+}$ with that induced by Fe$^{2+}$ (1–4).

The structure that in yeast frataxin a trimer is the basic building block of larger oligomers, while for the bacterial homologue a tetramer appears to form the basis for formation of higher order oligomers. These studies provide the basis for understanding the mechanisms of metal-induce frataxin oligomer assembly and give new insights into frataxin function in iron storage and detoxification. They also provide an insight into the interplay between frataxin and other proteins, to which iron is delivered. The structural details of the models also reveal striking similarities between frataxin and the evolutionary unrelated ferritin superfamily of iron storage proteins.

**References**


**SW02.S7–6**

An engineered binding protein targeting a critical region in the alpha-synuclein sequence

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Parkinson’s disease (PD) is the second most common neurodegenerative disease. Despite the fact that several studies have reported α-synuclein fibrillation as the main player in pathogenesis of PD and other synucleinopathies, its exact role in the disorders is not yet known. In this study, we aimed to select and characterize an engineered binding protein for α-synuclein as a molecular tool to investigate protein-protein interactions of α-synuclein and their impact on the aggregation mechanism. The binder, Z$_{syn69}$, was selected by phage display from a combinatorial protein library based on Z$_{Aβ3}$, an engineered binding protein for Aβ. Z$_{syn69}$ binds to α-synuclein with affinity in the nanomolar range as determined by ITC. α-synuclein fibrillation, monitored by Thioflavin T, was strongly inhibited by Z$_{syn69}$. The structural basis of the α-synuclein: Z$_{syn69}$ interaction was investigated by NMR spectroscopy. Our results reveal a sequence region with a critical role for α-synuclein aggregation.

**SW02.S7–7**

Structural and functional features of ceruloplasmin in complexes with other proteins of acute phase

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Ceruloplasmin (Cp) is a copper protein performing multiple important functions in human organisms. Cp oxidizes highly toxic ferrous ions to ferric state for further incorporation into apo-transferrins, catalyzes Cu(I) oxidation and oxidation of bio- genic and synthetic amines. As an efficient antioxidant Cp prevents oxidative damage to proteins, DNA and lipids. Membrane-bound Cp found in astrocytes forms a complex with ferroportin 1, thus regulating iron level in central nervous system and preventing free radical reactions. Interactions of Cp with other proteins further widen the range of its functions and structural characterization of such complexes provides important functional insights. The proteins involved, including Cp itself, are notoriously flexible and glycosylated, which makes the high resolution studies extremely difficult. We employed a multidisciplinary approach to study macromolecular assemblies of Cp with cat ionic proteins of neutrophils, such as myeloperoxidase (Mpo) and lactoferrin (Lf). These complexes exist in vivo and were identified previously in samples of serum and purulent exudates from...
patients with various inflammatory diseases. It was supposed that the capability of Cp to form complexes with these cationic proteins might be part of a regulatory mechanism in the pathogenesis of systemic vasculitis. Lf is a multifunctional glycoprotein abundant in biological fluids and tissues. It maintains iron homeostasis, possesses wide-range antimicrobial activity and immunomodulatory properties, and is able to act as an antioxidant agent and transcriptional factor. The Cp-Lf complex is expected to play a role in iron metabolism as Cp ferroxidase activity is enhanced in the presence of Lf. The ability of Cp to interact with myeloperoxidase (Mpo) and to inhibit its prooxidant properties likely imparts it with additional antioxidant activity in vivo. Mpo is one of the major proteins of the antimicrobial system of mammalian neutrophils, involved in a cascade production of RNOS (reactive nitrogen and oxygen species). However, excessive RNOS are deleterious for cells and tissues, as they attack biomolecules and change their structure and function. Chronic excessive RNOS production by neutrophils is involved in the onset of many diseases, including atherosclerosis. Therefore, modulation of RNOS production might be applicable for treatment or prevention of inflammatory diseases. We established the stoichiometries of binary and ternary Cp-Lf, Cp-Mpo and Cp-Lf-Mpo and report the first crystal structure of the Cp-Mpo complex at 4.7 Å. Small angle X-ray scattering (SAXS) was employed to validate this model and to construct low-resolution models of Cp-Lf and 2Lf-2Cp-Mpo complexes in solution. The structure of Cp with free labile sites at 2.6 Å was employed for molecular modeling and for discovering new details of Cp spatial organization. Our X-ray Cp-Mpo structure provides insights into the mechanism employed by Cp to inhibit ClO production by Mpo. It also shows details of the machinery underlying the oxidase activity of Cp towards different substrates. Structural data obtained suggest that Mpo protects the antioxidant properties of Cp by shielding its sensitive loop from proteases. Integrity of the loops important for incorporation of Fe3+ into Lf, which activates the ferroxidase activity of Cp. A model of the ternary complexMpo-2Cp-2Lf constructed using our SAXS data supports this mechanism. Occurrence of such complexes in inflammation foci is likely to extinguate the results of respiratory burst in neutrophils or to prevent the origination of Cp derivatives (proteolytic fragments) possessing pro-oxidant activity. The models obtained here elucidate the mechanism by which the very formation of a complex of Cp with its partner(s) enforces the antioxidant activity of this copper enzyme.

**SW02.S7–9**

**The structure of a membrane-bound sodium pumping pyrophosphatase**

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Membrane-bound pyrophosphatases (M-PPases) couple pyrophosphate hydrolysis or synthesis to Na+ or H+ pumping. They are found in plants, bacteria and protozoans and are crucial for survival in various stress condition (such as low light intensity, anoxia, cold and mineral deficiency) and are also important for plant maturation. We have solved the metal bound resting state (TmPPase:CaMg) and product bound (TmPPase:MgPi) structures of *Thermotoga maritima* sodium pumping M-PPase (TmPPase) at 2.6 Å and 4 Å resolution, respectively. The resting state structure shows an open active site cavity with the hydrolytic center at the top (20 Å above the membrane), followed by a ‘coupling funnel’ formed by conserved, charged residues lining six a-helices. The ‘coupling funnel’ ends at the gate formed by the conserved Asp243, Glu246 and Lys707 and below this is an exit channel leading to the periplasmic space. Comparison of our two Tm-PPase structures with the recently solved crystal structure of *Vigna radiata* H+-pumping pyrophosphatase in the state with product analogue bound (VrpPase:PNP) shows movement of the helix 12 in VrpPase:PNP and TmPase:MgPi. We presume that upon substrate binding a transient state is formed in which sliding of enzymes are widely distributed throughout the bacterial domain of life and are believed to have originated billions of years (Gyr) ago [3]. Here, we report the resurrection of Precambrian β-lactamas corresponding to several nodes in the phylogeny of Gram-negative bacteria. Despite extensive sequence differences between ancient and modern enzymes (on the order of one hundred amino acid differences), the resurrected proteins properly fold into the canonical lactamase structure. The ancient enzymes display high temperature stability, having denaturation temperature enhancements up to about 35°C with respect to modern β-lactamas. The most ancient of these β-lactamas are promiscuous towards a variety of substrates and are able to endow modern microorganisms with resistance towards different types of antibiotics (including third-generation) at levels that are comparable to those found in clinical isolates. This enhanced promiscuity is not accompanied by significant changes in the active-site region as seen in static X-ray structures, suggesting a plausible role for long-range dynamics in the evolution of function in these proteins. Two to three billion-years-old β-lactamas are also able to endow modern microorganisms with significant levels of resistance towards a variety of antibiotics, opening up the possibility of performing laboratory replays of the molecular tape of lactamase evolution. Overall, this work supports the findings that Precambrian resurrections generate hyper-stable proteins, provides direct evidence for the generalist-to-specialist conversion during protein evolution and opens up new possibilities in the study of the emergence of antibiotic resistance.

**References**


the helix 12 towards the periplasm/vacuolar lumen opens the gate and the exit channel and leads to ion pumping. Superimposing helices 3–6, 9–12 and 13–16 of TmPPase suggests that M-PPases arose through gene triplication.

**SW02.S7–10**

**Crystal structure and mutational analysis of thermostable direct hemolysin from *Grimontia hollisae* reveals new insights on membrane binding and physiological activity**

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Thermostable direct hemolysin (TDH), a bacterial pore-forming toxin, lyases red blood cells and biological membranes. To elucidate the structural basis of pore formation, three-dimensional structure of the TDH from *Grimontia hollisae* (Gh-TDH) has been solved at 2.1 Å resolution. In contrast to the symmetric C4 tetramer structure of the TDH from *V. parahaemolyticus*, the Gh-TDH forms dimer of dimers overall structure and crystallizes in the orthorhombic space group P2_{1}2_{1}2_{1}. The crystal packing of the Gh-TDH adopts three possible arrangements with minor differences which are mainly derived from the inter-subunit interactions between the phenolic group of Tyr^{53} and the phenyl group of Phe^{159} as well as between the amino side chain of Lys^{97} and the amide carbonyl side chain of Gln^{104}. The Gh-TDH exhibits full, partial, and inactive pore-forming toxicity when exists in tetramer, dimer, and monomer conformation, respectively, supporting the dimer as a minimal unit of structural basis for physiological activity. Site-directed mutagenesis and flow cytometry analyses of both the N-terminal truncation and the solvent-exposed aromatic residues show that initial binding of the Gh-TDH to the membranes is promoted at least by Trp^{65} and Tyr^{87} and that the N-terminal helix and both the Trp^{39} and Tyr^{107} residues are related to the post-binding process of hemolytic and cytotoxic activities. Taken together, these findings provide new insights into TDH’s actions in membrane recognition/binding, insertion, hemolytic activity, and cytotoxicity.

**SW02.S7–11**

**On the possibility of lipid-induced regulation of conformation and immunogenicity of hemagglutinin from influenza A virus H1/N1 in the content of TI-complexes**

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The tubular immunostimulating complex (TI-complex) consisting of triterpene glycoside cucumarioside A2-2, cholesterol and glycolipid monogalactosyldiacylglycerol (MGDG) from marine macrophytes is one of the most perspective antigen delivery system for the creation of safe and high-performance subunit vaccines. TI-complexes can incorporate amphiphilic antigen and provide adjuvant effect. TI-complexes have much more pronounced immune response than classic ISCOMs, liposomes or Freund’s complete adjuvant (Kostetsky et al., 2011).

MGDG performs the role of a lipid matrix for the protein antigen, thus modifications of its physicochemical properties can be used to alter antigen conformation and therefore to enhance immunogenicity of whole vaccine construction (Sanina et al., 2012).

Physical state of MGDG depends on its fatty acid composition, which in turn varies in dependence of taxonomic position of marine macrophytes (Sanina et al., 2004, 2008). The aim of the present work was to study the influence of MGDGs, isolated from three species of marine macrophytes (*Sargassum pallidum*, *Ulva fenestrata* and *Zostera marina*), on conformation and immunogenicity of recombinant monomeric hemagglutinin of Influenza A virus H1/N1 *A/California/07/2009* as a model antigen for subunit vaccine based on TI-complexes.

MGDG’s influence on conformation of hemagglutinin was studied by means of differential scanning calorimetry, fluorescence spectroscopy and circular dichroism. To analyse immunogenicity of TI-complexes with incorporated hemagglutinin, mice were immunized subcutaneously twice, at an interval of 14 days. Experiments were terminated 21 days after the first immunization.

Results showed that in spite of significantly different effect of MGDGs on conformation of antigen, the level of anti-hemagglutinin antibodies similarly increased by 2 times compared with HA alone independently on the source of glycolipid. The lack of correlation between effects of different MGDG samples on conformation and immunogenicity of recombinant monomeric hemagglutinin of Influenza A virus H1/N1 *A/California/07/2009* was occurred because chosen antigen unlike trimeric hemagglutinin comprises the sequential determinants only, whose presentation to immune system do not depend on conformation of protein.

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SW02.S7–12
Structural insights into spatial organization and mechanism of DNA-binding of histone-like HU-proteins from mycoplasmas
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HU proteins are the most abundant DNA-binding proteins in bacteria. They play a role in DNA repair, DNA recombination and DNA replication. Noteworthy, HU proteins were found only in prokaryotic organisms but not in eukaryotic. Moreover, HU protein deletion from the bacteria genome is lethal in many cases.

Mycoplasmas are the smallest known microorganisms and intercellular parasites, which cause various mammal diseases. Due to drastically reduced genome sizes and subsequent lack of mismatch reparation system HU protein deletion from these organisms is lethal. Structures of mycoplasma HU proteins as well as their complexes with the bounded DNA could provide an insight into the mechanism of HU recognition of non-canonical DNA structures that enables DNA recombination and repair. Also this could lead to development of new therapeutic agents selectively blocking DNA binding with HU proteins of pathogenic organisms with subsequent cell death. Up to date there is no structural information on HU proteins from mycoplasmas.

In the present work we have selected two HU proteins from mycoplasmas Mycoplasma gallisepticum (poultry parasite) and Spiroplasma melli ferum (insect parasite). Both proteins have been successfully expressed in E. coli and purified. Crystallization screening has been carried out and crystals were obtained for both proteins. Crystals of HU protein from Mycoplasma gallisepticum gave diffraction about 3.3Å, however for the protein from Spiroplasma melli ferum 1.4Å structural data were obtained. This allowed us to solve the structure of mycoplasma HU protein for the first time. In addition, we have synthesized several oligonucleotides differing in size, mismatch type, bp composition, etc. mimicking real DNA and confirmed their binding with recombinant HU proteins from mycoplasmas. Attempts to crystallize complexes of HU proteins with these DNA-fragments are now in progress.

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SW02.S7–14
Influence of stress-changed lipids on conformation of OmpF-like porin of Yersinia pseudotuberculosis
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It is assumed that compensatory changes in physicochemical properties of membrane lipids are necessary to optimize conformation and thereby functional properties of membrane proteins at the new environmental conditions. In spite of the high ability of Yersinia pseudotuberculosis to adapt to different environmental conditions, the effect of different lipid surroundings on conformation of the major membrane protein - OmpF-like porin (YopF) is still unclear. Various stress factors such as the heat shock, low pH of medium, the treatment by the phenol biocide was shown to increase drastically the content of lysophosphatidyl-lethanolamine (LPE) in membranes of bacteria. Present work was aimed to compare effects of different content of 'stress lipid' LPE in lipids derived from Y. pseudotuberculosis cells exposed and non-exposed to phenol on conformation of OmpF-like porin of these bacteria.

Phenol treatment of Y. pseudotuberculosis increased the level of LPE by 2.5-times compared with cells untreated by phenol. The simultaneous two-fold increase of saturation in fatty acid residues resulted in increase of phase transition temperature of lipids from 17 to 40°C. Results of DSC showed more stabilizing effect of lipids enriched by LPE to the thermal denaturation of porin compared with lipids contained small amount of LPE. In turn, deconvolution of the intrinsic fluorescence spectra have shown that different thermostabilizing effect of these lipids connects with their different influence on the tertiary structure of porin.

Integral conformational rearrangement of protein was supported by drastic changes in microenvironment of tryptophan residues, likely resulted in convergence of monomers in trimeric porin and due to exposure of outer tryptophan residues to the
water environment. These rearrangements in conformation of YOmpF may impede the porin channel permeability at the stress conditions for bacteria.

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SW02.S7–15
Conformational change of Starmaker protein and IST ability of calcium ions binding is crucial for biomineralization activity
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Biomineral structures, like bones, teeth and shells are formed under strict biological control. A fish otolith is a biomineral responsible for sensing of gravity. Otolith formation is controlled by acidic proteins, which frequently belong to a group of intrinsically disordered proteins (IDPs). They are dynamic and heterogeneous population of molecules without a well-defined folded structure. Starmaker (Stm) protein is an IDP, which controls shape, size and polymorph of otoliths in zebrafish.

We applied circular dichroism (CD), gel filtration, single molecule FRET (smFRET) and calcium ions binding assay to examine relationship between structure and function of Stm. Stm binds calcium ions. It is strongly extended, rod-shaped molecule and it undergoes significant compaction in the presence of calcium ions in the range of 0–100 mM. The Stokes radius change of 11–34 Å. Surprisingly, CD analysis did not show changes in Stm secondary structure in the presence of the same range of Ca2+ concentration. smFRET measurements of Stm mutant labeled with donor and acceptor of fluorescence at the N-terminus has shown that it undergoes compaction in this region. Based on above results we assumed that Stm extended conformation facilitates calcium ions binding, while the compaction of the molecule induced by the ions causes formation of PILP (polymer induced liquid precursor) in the process of calcium carbonate crystal formation.

Structural studies of proteins involved in biomineralization lead to better understanding of mechanism of biomineral formation. Naturally occurring biominerals differ from inorganic crystals. If we understand the role of proteins in biomineral formation, we will be able to design and produce new materials with practical use in medicine and industry.

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SW02.S7–16
Unraveling the determinants of polyketide synthases (PKS) substrate specificity
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Introduction: Polyketides are secondary metabolites produced by bacteria, plants and fungi. They constitute a very heterogeneous group of compounds. Some of them play an essential role in biological processes (e.g. cell wall biogenesis, pathogenesis), others display pharmacological (e.g. bactericidal, fungicidal, immunosuppressive or anti-tumoral) properties. Polyketide synthases (PKS) are thus considered as powerful enzymes for pharmaceutical applications for humans and as potential therapeutic targets against bacterial pathogens. Nevertheless understanding of molecular bases of their programming (substrate specificity, stereochemistry and reaction cycle) remain deeply unknown.

Here we deciphered the determinants of PKS substrate specificity from our study on mycobacterial PKS representatives, Mas and PpsC, synthases of mycocerosic acid and of (phenol)phthiocerol, using respectively methyl-malonate and malonate as an extender unit in condensation reaction.

Study Objectives: The aim of the study was to identify specificity determinants of PKS from Mycobacterium tuberculosis.

Methods: Alignment of protein primary structures was performed using BlastP. Structural analysis conducted using ClustalW. Point mutations were introduced by site-directed mutagenesis. His-tagged proteins were produced and purified to homogeneity by IMAC. Enzyme specificity was tested by SDS-PAGE using (13C) radiolabelled substrates.

Results: Numerous mutations were introduced into the structure of Mas and PpsC acyltransferase domain, responsible for the substrate binding. Generated mutations changed biophysical characteristics of AT-Mas and AT-PpsC and modulated their substrate affinity. We identified several key amino acids determining PKS substrate specificity, divergent for both studied proteins: M624, S726, N776 and L777 for Mas and R578, A665 and M816 for PpsC. Obtained results were confirmed for the whole proteins, which led to modulation of their substrate affinity. Thus, the determinants of the PKS specificity were identified.

SW02.S7–17
Oxidative modification of fibrinogen
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Oxidative modification of proteins may reflect the dysfunctions in pro-/antioxidant homeostasis. The amino acid side chain carbonylation, methionin sulfoxide and bithyrosin formation, and thiol reduction are typical consequences of reactive oxygen species (ROS) attack on proteins. Measuring of plasma proteins carbonylation is broadly used approach for quantifying oxidative damage on proteins and qualifying red-ox status of the whole organism. Several plasma proteins recently were considered as antioxidants due to their high potential to accept and suppress the ROS attack. Fibrinogen is among such proteins. Its central role in hemostasis is well known. This should make a blood coagulation process highly susceptible to ROS.

We have shown that the treatment of fibrinogen with Fe/H2O2 system reflected with its highly increased carbonylation, on the other hand the level of fibrin oxidation treated with the same agents was negligible. This data clearly shows that the main target of oxidants is fibrinopeptides and not a des-AB-fibrin (fibrin monomer) as it was suggested earlier.

SW02.S7–18
Metal-specific structural response of parvalbumin to the binding of physiological cations
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Parvalbumin (PA) is a small cytosolic protein of the EF-hand superfamily. It is expressed by vertebrates in fast-twitch muscle cells, specific neurons of the central and peripheral nervous system, sensory cells of the mammalian auditory organ and some other cells. PA binds different metal cations, but only Ca2+- and
Mg\textsuperscript{2+}-loaded forms are of undoubted physiological significance. Although the main role of the protein is considered to be metal buffering, the exact function of PA in neuronal and many other tissues is still mainly hypothetical. Moreover, recent evidences indicate that PA also fulfills more complicated functions, which may be determined by the diversity in structural changes in response to the binding of different metal cations. Thus, the study of metal-induced conformational changes is important for understanding of the physiological function of PA.

In the present work the conformations of \( \alpha \) and \( \beta \) isoforms of pike PA in the Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-loaded state were studied by intrinsic fluorescence, circular dichroism and bis-ANS extrinsic fluorescence. The metal-binding sites of both isoforms of pike PAs do not differ significantly in affinity for Ca\textsuperscript{2+} and Mg\textsuperscript{2+}. However, the response to metal-binding is isoform-specific. Our data reveal similarity of the Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-induced conformational changes in \( \alpha \)-parvalbumin. In contrast, all experimental approaches employed in the present work indicate the existence of differences between Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-bound forms of \( \beta \)-PA.

We have determined the structural region causing different spectral response on the binding of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} ions in pike \( \beta \)-parvalbumin. Importantly, the same region was shown to be the most sensitive to the changing of cation environments in \( \beta \)-PAs of other species. We also discuss the possible physiological consequences of the structural rearrangements accompanied Mg\textsuperscript{2+}/Ca\textsuperscript{2+} exchange in pike \( \beta \)-PA. Overall, the present work supports the suggestion about the sensor role of parvalbumin.

**SW02.S7–19**

Carbonylation: effects of structural and functional modifications of fibrinogen on endothelial cells

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Oxidative modifications are relevant in physiological processes, contributing also to protein modification and cellular damage. Carbonylation, an irreversible protein oxidation, can alter protein structure hence modifying their function. Carboxyl groups are introduced in proteins at the side chains of lysine, arginine, proline and threonine residues, producing ketone or aldehyde derivatives. Increased levels of protein carbonyls, widely measured markers of oxidative stress, have been found in several acute and chronic disorders, including cardiovascular diseases (1). Our interest focused on fibrinogen whose function is central to haemostasis. The aim of this study was to investigate the effects of carbonylation on fibrinogen stability, and to correlate its structure alteration to its function on Human Umbilical Vein Endothelial Cells (HUVEC).

To reach this goal we used the following methodological approach:

ROS generation was induced by 2,2’-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) used at different concentrations (0.01–1 mM). Fibrinogen protein carbonyl content was estimated by a fluorometric assay. To assess any modification in the clotting activity of carbonylated fibrinogen, fibrinogen polymerization at 595 nm at room temperature was monitored. A reduction in the absorbance values (from 100% of the control fibrinogen sample absorption to 3% of the maximum carbonylated fibrinogen sample), together with an increase in carbonyl content – 4.76 fold compared to control – were observed. As suggested by circular dichroism spectroscopy, the structural features in the carbonylated samples were markedly different compared to those of uncarbonylated controls. The incubation of carbonylated fibrinogen with HUVEC resulted in a significant reduction in cell viability estimated by mitochondrial activity.

In conclusion, an increase in fibrinogen carbonylation reflects a slowdown clotting activity of fibrinogen, due to an alteration of the protein structure and a marked impairment in cell viability.

Studies are in progress to understand the molecular mechanisms underlying these effects.

**Reference**


**SW02.S7–20**

A new approach on protein folding correction: rescue of arginine to cysteine mutations using thiol compounds

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Most proteins to be biologically active must present a correct 3D arrangement, determined by the amino acid sequence. Proper folding is acquired by interactions established between specific residues which nucleate and trigger the cooperative folding reaction into the final topology, corresponding to the most stable structure under physiological conditions. One half of all sequence alterations in genetic diseases are missense mutations. These can affect the structure, stability and folding of the mutant protein. Rescue of proper folding by chemical chaperones has been extensively studied in the last years. Cystathionine \( \beta \)-Synthase (CBS) deficiency, characterized as a conformational disorder, has been a target for folding rescue by the use these compounds.

We aimed to study the effect of the thiol compounds Cysteaminic (CySH) and Mercaptoethylguanidine (MEG) in the specific rescue of Arginine (R) to Cysteine (C) mutations. To this end we studied the common CBS mutation R336C. We postulate that these thiols will bind to the mutant residue C forming a structure that resembles the wild-type (WT) residue R thus restoring enzyme activity.

We used purified proteins produced in \( E. \) coli. As negative controls the WT and R336H protein were used. To test the specificity for CySH and MEG Cysteine (Cys) was used as a negative control. Purified proteins were pre-incubated with CySH, MEG or Cys followed by evaluation of CBS activity and thermal inactivation profile. Differential scanning fluorimetry assays were performed to monitor protein thermostability.

For the R336C protein, all tested conditions showed an increase in activity. A six-fold and 17-fold increase were observed when samples were incubated for 7 h with 0.25 mM CySH and 1 mM MEG, respectively, at 37°C, while no effect was observed for the R336H or WT proteins. Cys was unable to rescue any of the studied proteins.

Our results support the hypothesis that these compounds specifically rescue proteins with an R to C mutation.
SW02.S7–21
Deciphering the mechanism of yeast Nth1 activation
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Neutral trehalase (NTH1) (EC 3.2.1.28) is a yeast enzyme, which hydrolyzes trehalose to two glucose molecules. z-z-trehalose (1-z-D-glucopyranosyl z-D-glucopyranoside) plays an important role as a reserve and stress metabolite in yeast cells. According to former results, the activity of Nth1 is mediated by the yeast isoforms of 14-3-3 proteins - Bmh1 and Bmh2 [1]. Nth1 is phosphorylated by PKA on multiple sites forming a complex with Bmh protein in the ratio 1:2, which leads to the activation of Nth1. Limited proteolysis confirmed that the 14-3-3 proteins interact with the N-terminal segment of Nth1, where all phosphorylation sites are located. Site-directed mutagenesis together with enzyme activity measurements and the activation studies of mutant forms revealed that Ser60 and Ser83 are important for PKA-dependent and 14-3-3-protein-mediated activation of Nth1.

[2]. Hydrogen/deuterium exchange (HDX-MS) coupled to mass spectrometry was used to identify structural changes which occur upon the interaction between Nth1 and Bmh1. Our results show that 14-3-3 protein binding affects the structure of several Nth1 regions including those surrounding the active site suggesting that regions surrounding the buried active site undergo a structural change upon the complex formation of Nth1 with Bmh1. The interaction surface of Bmh1 includes not only the surface of the ligand binding groove where the phosphorylated N-terminal segment of Nth1 binds but also surfaces outside the central cavity of Bmh1 dimer. Similar binding surface was recently described for the human 14-3-3_8 in its complex with the regulator of G-protein signaling 3 using HDX-MS [3]. Supported by the Grant P207/11/0455 of the Grant Agency of the Czech Republic.

References

SW02.S7–22
Comparison of protein redox homeostasis parameters in myocardial tissue of α-galactose induced and naturally aged rats
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Background: The aging related myocardial dysfunction has important implications with regard to impaired redox homeosta-

dis. Current study focused on protein oxidation and other oxidative damage markers as well as antioxidant status in myocardial tissue of mimetic aged rats, naturally aged rats and their respective young control group.

Methods: Intraperitoneal injection of α-galactose (60 mg/kg/day) for 6 weeks to young male Wistar rats (20-week-old) was used to establish mimetic-aging model. In the current study, we investigated the myocardial tissue levels of advanced oxidation protein products (AOPP), protein carbonyl groups (PCO), various tissue thiol fractions like total (T-SH), protein (P-SH), non-protein thiol groups (NP-SH), and lipid oxidation parameters such as lipid hydroperoxides (LHP). Our study was also covered superoxide dismutase activity (Cu-Zn SOD) and ferrous reducing antioxidant power (FRAP) parameters. Western blot procedure was used to evaluate protein redox status in myocardial tissue samples.

Results: In α-galactose induced aged rats PCO concentrations were significantly higher than young control group (p < 0.05), whereas T-SH, P-SH, and NP-SH levels were not significantly different. Cu-Zn SOD activities in both experimental groups were significantly lower than the corresponding young controls (p < 0.01, for both groups). In addition, LHP concentrations were only found to be different between mimetic-aging and control group (p < 0.05). In naturally aged rats AOPP levels were found to be significantly higher (p < 0.05), T-SH, P-SH, NP-SH and FRAP concentrations were not different than young controls.

Conclusions: Our results that those in the mimetic aging group share significant similarities in terms of impaired protein redox status with the naturally aged rats and may be considered as a reliable experimental model for myocardial aging.

SW02.S7–23
Analysis of additional lipids variation effect on the crystallization in meso
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Membrane proteins play crucial role in many processes of inter-cellular communications, mass transport, energy transfer inside our bodies. Unfortunately, nowadays there is no much data about their high-resolution structure that could help to make new medicine targeted on their dysfunctions that are common reasons for many severe diseases.

The X-Ray diffraction analysis is one of the best methods to get high-resolution structure of a membrane protein.

For the X-ray membrane protein diffraction analysis it is necessary to get protein crystals with good diffraction properties. Some good results are present (e.g. [3]) but it is still insufficient.

One of the most promising method for membrane protein crystallisation is the in meso [4] method which is based on protein integration into lipidic mesophase, basically cubic and sponge, and then inducing the nucleation process by adding precipitants and some additives.

Crystallization of membrane proteins in meso is an outstanding challenge for biophysicists. It is necessary to collect the data about optimal conditions for crystal growth. And also one should be sure that the protein in crystal has the same functionality as in vivo. Natural lipid environment is one of the most crucial factors determining the protein functionality. Moreover, sometimes it is impossible to isolate a membrane protein without annular lipids. But how it affects the crystal growth? We have
carried out an investigation of several lipid additives influence on crystallization in meso with the help of electron microscopy, small angle X-Ray scattering and X-Ray diffraction.

References

SW02.S7–24
Novel structural studies on the PII-signaling system
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PII, a highly conserved homotrimeric signaling protein, interacts with target proteins via its flexible T-loop. ADP, ATP, 2-oxoglutarate and postranslational modifications affect this loop and PII function. In all life domains, PII plays key roles for adaptation to changes in nitrogen, carbon and energy abundance. We shed light on PII adaptation to high salinity by reporting crystal structures of PII from Haloferax mediterranei, an extreme halophilic archaea in which PII interacts directly with glutamine synthetase [1]. We determine structures of PII bound to ATP or ADP+ 2-oxoglutarate. As expected for a halophile, short and acidic residues prevail. A unique N-terminal extension forming a crown over the hemispheric PII body, and an unusual z-helical fold of the T-loop, are observed in these structures.

No structural information exists on the consequences of T-loop postranslational modification. We are studying the PII-AmtR system of Corynebacterium glutamicum, where AmtR, a repressor and master regulator of nitrogen control of gene expression, binds to adenyllylated PII [2]. We now report the crystal structure of AmtR in an inactive form that is likely the one binding to PII, showing a homodimeric TetR fold. It is unclear how a homodimer can interact with a trimere. The higher potential of both proteins’ surfaces only allows their interaction through the DNA-binding region of AmtR. The PII adenyllylated group might prevent DNA binding by mimicking a DNA nucleotide.

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References

SW02.S7–25
Interaction of the cisplatin with the sodium potassium pump
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Sodium potassium pump [Na+ / K+-ATPase] creates the sodium and potassium gradients across plasma membrane in cells of all animals. This member of P-type ATPases pumps three sodium ions out and two potassium ions into the cell for each ATP molecule and maintains their electrochemical potential gradients, required for electrical excitability and transport of other ions, nutrients, and neurotransmitters, as well as for regulation of cell volume and intracellular pH. Change of activity of this enzyme can influence these mechanisms and can potentially result in variety of diseases.

Cisplatin [cis-diaminedichloroplatinum(II)] is a chemotherapy drug, which is used to treat various types of cancer. The way that cisplatin operates is by forming a platinum complex inside of a cell which binds to DNA and impairs DNA replication and cell division. However, treatment by the cisplatin has many side-effects such as hearing loss, neuropathies and acute renal failure. For the correct functioning of the kidneys concentrations of sodium ions is essential. Key role in maintaining of the concentration of sodium ions is played by sodium potassium pump.

We tried to verify the hypothesis, that acute renal failure during the treatment by the cisplatin can be caused by interaction of the cisplatin with the sodium potassium pump. According to our results on whole protein and on its large cytoplasmic look [C45] we can conclude that the cisplatin significantly inhibits activity of the sodium potassium pump. The next conclusion is that cisplatin binds to cysteine residues in the large cytoplasmic loop and the molecular mechanism of the inhibition might be related to cisplatin binding to Cys367 near the phosphorylation site.

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SW02.S7–26
Age related variations in oxidative damage markers in tissue of rat prostate
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Introduction: Aging represents a major risk factor for prostate cancer; however, redox regulation mechanisms responsible for this relationship remain unclear. In the present study we hypothesized that an aging-related impairment of redox homeostasis in prostate tissue may be responsible, in part, for enhanced levels of oxidative stress and, thus, play a role in prostate cancer development.

Methods: Inbred male Wistar rats were divided into two groups according to age; as young (5 months) and old (24 months). In the current study, we investigated the prostate tissue levels of advanced oxidation protein products (AOPP), protein carbonyl groups (PCO), various tissue thiol fractions like total (T-SH),...
protein (P-SH), non-protein thiol groups (NP-SH), and lipid oxidation parameters such as lipid hydroperoxides (LHP). Our study also covered superoxide dismutase activity (Cu-Zn SOD) and ferrous reducing antioxidant power (FRAP) parameters. Western blot procedure was used to evaluate protein redox status in prostate tissue samples.

**Results:** AOOP, PCO, NP-SH, and LHP levels of aged rats were significantly higher than those of the young rat group (p < 0.01, p < 0.05, p < 0.001, p < 0.05, respectively). On the other hand, all T-SH, P-SH and FRAP levels were not found to be different (p > 0.05). In addition, Cu-Zn SOD activities in experimental group were significantly lower than the ones in corresponding young controls (p < 0.05).

**Conclusion:** We suggest that increased Np-SH levels found in aged rats may point to an adaptive reaction to oxidative protein damage, reflecting AOOP and PCO overproduction. We are of the conviction that the increased oxidative protein damage markers and decreased Cu-Zn SOD activity that we determined in aged male rats are a risk factor for the development of prostate cancer. Additional studies are warranted to clarify the potential involvement of these changes as mechanistic factors in the association of aging with prostate cancer risk.

**SW02.S7–28**

**High molecular weight forms of human phenylalanine hydroxylase: the role of the ACT domain in the balance between a fully functional protein and the large inactive aggregates**

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Protein misfolding results in protein aggregation or accelerated degradation and it is the culprit in numerous human diseases classified as Conformational Disorders. Phenylketonuria (PKU) is a loss-of-function disorder caused by misfolding of the cytosolic protein phenylalanine hydroxylase (hPAH). A large number of point mutations in hPAH leads to misfolded proteins with a high propensity to aggregate and form higher-order oligomers and large inactive aggregates when expressed in vitro and to be rapidly degraded in vivo. It became evident that PKU mutations throughout the entire sequence of the protein (regulatory, catalytic and oligomerization domains) have a negative impact upon the regulatory domain (RD), which seems to play a crucial role in the instability and misfolding of the protein [1]. This domain contains a βββββ motif (ACT domain) that in other proteins is always involved in oligomerization and small molecule binding processes. However, in the native hPAH tetramer structure there is no contact between the four ACT domains. Recently we were able to isolate a high molecular weight species of hPAH between the functional tetramer and the inactive large aggregates, with impaired regulatory and stability properties. We hypothesized that this species could be formed by assembly of two tetramers via the ACT domain, and mutations in the regulatory domain or elsewhere in the protein impacting the RD could increase the propensity to form higher order oligomers and aggregates and thus contributing to the loss of the functional form. Understanding the contribution of the regulatory domain of the protein to the PKU disorder is crucial to design new strategies to rescue the severe forms of the disease by pharmacological chaperoning.

**Reference**


**SW02.S7–29**

**The evaluation of protein redox status in gastrocnemius and soleus muscles of mimetic and naturally aged rats**


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**Background:** Aging is accompanied by a progressive loss of skeletal muscle mass and strength, known as sarcopenia, which leads to reduced functional capacity and an increased risk of developing chronic metabolic disease. The substantially atrophy is most commonly seen in gastrocnemius (fast twitch fibers) and less commonly observed in slow twitch fibers. Specifically, the rat soleus muscle...
muscle (predominantly slow twitch fibers) exhibits a strikingly similar rate of atrophy in senescence as the gastrocnemius muscle. Current study focused on the levels of protein oxidation markers in gastrocnemius and soleus muscles of mimetic aged rats, naturally aged rats and their respective young control group.

**Methods:** Intraperitoneal injection of L-galactose (60 mg/kg/day) for 6 weeks to young male Sprague–Dawley rats (20-week-old) was used to establish mimetic-aging model. In the current study, we investigated the muscle tissue levels of advanced oxidation protein products (AOPP), protein carbonyl groups (PCO), total thiol (T-SH) and protein thiol groups (P-SH) Western blot procedure was used to evaluate protein redox status in muscle tissue samples.

**Results:** Gastrocnemius: In mimetic aged rats PCO concentrations were significantly higher than young control group (p < 0.05), whereas AOPP, T-SH and P-SH levels were not significantly different. PCO and AOPP levels in naturally aged group were significantly higher than the corresponding young controls (p < 0.05, p < 0.001, respectively). On the other hand, T-SH and P-SH levels were not significantly different between naturally aged and controls.

Soleus: In mimetic aged rats PCO and AOPP concentrations were significantly higher than young control group (p < 0.001, p < 0.05 respectively), whereas T-SH and P-SH levels were significantly lower than young controls (p < 0.05). PCO and AOPP levels in naturally aged group were significantly higher than the corresponding young controls (p < 0.05). In addition, the levels of thiol groups in naturally aged group were not significantly lower than controls.

**Conclusions:** Our results show that gastrocnemius muscle of naturally aged group more susceptible to oxidative damage than soleus. However, soleus muscle much more vulnerable to L-galactose induced oxidative stress than gastrocnemius. Soleus rich in mitochondria and consumes more oxygen due to the characteristic energetic metabolism of slow-twitch fibers. L-galactose shows its free radical inducing effects particularly on mitochondria. Because of its effect we suggest that soleus muscle may be more affected by L-galactose application. Our current results due to mimetic aging model encourage mitochondrial oxidative stress theory of aging. If L-galactose was used optimal time and dosage it would be useful for the establishment of model aging. Finally, we hope to optimize the current condition for our upcoming research.

**SW02.S7–30**

**Preliminary characterization studies on recombinant protein FKBP39 from Drosophila melanogaster**

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Juvenile hormone (JH) and ecdysteroids regulate a wide variety of developmental and physiological processes in insects. Two proteins, immunophilin FKBP39 and calponin-like protein Chd64, that bind to DmUHRE1 (Drosophila melanogaster JH response element) were previously identified. It is was shown that FKBP39 and Chd64 can interact with each other and with ecdysteroid receptor, ultraspiracle nuclear receptor and methoprene-tolerant protein. This suggests that FKBP39 and Chd64 probably play important roles in cross-talk between JH and ecdysteroids.

To facilitate the exploration of the interrelationship between function and molecular properties of FKBP39, we elaborated and optimized a protocol for the efficient expression in *Escherichia coli* and purification of this protein. The two-step purification procedure uses affinity chromatography (TALON® resin) and size-exclusion chromatography. The molecular mass value of recombinant FKBP39 determined using electrospray ionization (ESI) mass spectrometry (40696.6 ± 2 Da) was corresponded with theoretical value (40826.1 Da). Using a various array of biochemical methods we perform first structural characterization of FKBP39. These analyses with in silico studies indicate that FKBP39 exhibits some properties of intrinsically disordered proteins. This results enable further molecular and structural characterization of FKBP39 and may be helpful in understanding the role of this protein in regulation of JH-dependent gene expression.

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**SW02.S7–31**

**Product of starmaker-like gene from medaka (Oryzias latipes) is a member of intrinsically disordered proteins family**

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**Starmaker-like (stm-l) gene was identified in a large-scale random in situ hybridization screen in the medaka (Oryzias latipes) developing ear.** Its genomic structure and expression pattern is highly similar to zebrafish (Danio rerio) starmaker (stm) gene. Although there is not significant sequence similarity between stm-l and stm encoded proteins (Stm-l and Sim, respectively) it was suggested that Stm-l is involved in otholits biosynthesis like Sim described earlier [1]. However, the protein encoded by stm-l gene has not been characterized yet.

To facilitate exploration of the molecular basis of Stm-l protein function, we have elaborated and optimized a protocol for efficient expression and purification of the homogeneous nontagged Stm-l. Using a diverse array of biochemical and biophysical methods we carried out the first structural characterization of Stm-l. These analyses of recombinant Stm-l, along with in silico examinations, indicate that Stm-l, despite the lack of sequence similarity with Stm, exhibits properties of intrinsically disordered proteins (IDPs), just like it was shown for Stm [2]. Our results provide evidence that some factors like Ca2+ ions or osmolytes may affect the structure of Stm-l. Preliminary studies show that Stm-l controls size and shape of calcium carbonate crystals. These results pave the way for further studies of proteins involved in otholits biosynthesis and in the future may be helpful with understanding this process.

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**References**

SW02.S7–32
The preparation of homogenous recombinant Chd64 protein from Tribolium castaneum
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Larval development in insects is controlled by two hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH). The mechanism of action of the latter remains poorly understood. Two proteins, a calponin-like protein Chd64 and immunophilin FKBP39, which bind Drosophila melanogaster JH response element, were previously identified. It has been shown that the proteins can interact with ecdysteroid receptor, utraspiracle and methoprene-tolerant protein. According to a proposed model Chd64 and FKBP39 are a part of multiprotein complex that mediates a cross-talk between JH and 20E. We expect to elucidate the function of the protein by exploring its biochemical and biophysical properties. Researches on Chd64 from D. melanogaster are already carried out in our laboratory. To extend our studies to distantly related insects we developed a protocol for overexpression and purification of Chd64 from Tribolium castaneum. cDNA was cloned to different pQE80L plasmid vectors to receive recombinants in fusion with His-tag, Strep-tag and both of them. The proteins were overexpressed in Escherichia coli BL21(DE3) expression strain and in all cases they were found in a soluble fraction of bacterial proteins. The purification procedure was optimized for a derivative with His-tag on a C-end. A two-step purification protocol includes: metal affinity chromatography and gel filtration. The purity and homogeneity of obtained samples were confirmed using SDS-PAGE and Western blotting. The molecular mass (22126.6 ± 2 kDa) was determined using ESI-MS and was compatible with a theoretical value (22 127 kDa).

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SW02.S7–33
Expression and purification of the intrinsically disordered otolith matrix macromolecule-64
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Otoliths are biominerals found in the inner ear of fish. They are part of the gravity and linear acceleration detection system. Otoliths are composed of calcium carbonate and organic matrix, which plays crucial role in crystals formation. It has been shown that a lot of proteins involved in biominalerization belong to the group of intrinsically disordered protein (IDP). Structural disorder helps them to carry out their functions. An impaired control of biominalerization and in consequence defective crystals formation are reflected in behavioral effects.

Otolith matrix macromolecule-64 (OMM-64) protein found in the otoliths’ organic matrix is contained in high-molecular-weight aggregate (HMW) along with otolin-1, heparan sulfate glycosaminoglycans and other unidentified components. It has been previously reported that HMW aggregates control the polymorph of crystals inducing aragonite formation. Moreover neither OMM-64 nor otolin-1 itself induces aragonite in the same in vitro crystallization system. Te aim of our work is to investigate the structure and function of OMM-64 separately and in the presence of the other HMW aggregate components.

Recombinant, nontagged OMM-64 was obtained in Escherichia coli BL21(3DE)pLysS. Purification procedure consisted of five steps was developed. It included: ammonium sulfate precipitation, size exclusion chromatography, chromatography on hydroxyapatite, ion exchange chromatography and hydrophobic interaction chromatography. Homogenous OMM-64 deprived of signal peptide was obtained for structural and functional studies. Bioinformatical and experimental analyses indicated that OMM-64 belongs probably to a group of intrinsically disordered proteins are presented.

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SW02.S7–34
Flagellin glycosylation in Burkholderia cenocepacia
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Burkholderia cenocepacia belongs to Burkholderia cepacia complex (BCC), a group of Gram-negative bacteria comprising many important human opportunistic pathogens. In immunocompromised patients with underlying lung disease (e.g. cystic fibrosis, CF), infection with bacteria belonging to BCC causes development of fatal pneumonia and the prevalence of B. cenocepacia infections in CF patients grew rapidly in last couple of decades. B. cenocepacia bacteria are flagellated and flagella play an important role in their pathogenesis. Non-flagellated mutants show decreased adhesion to and invasion of epithelial cells as well as weaker induction of host inflammatory responses. Flagella also play a pivotal role in biofilm formation what is another important factor in B. cenocepacia pathogenicity. We analyzed the flagellin from B. cenocepacia K56-2, belonging to ET12 epidemic clone. Strains of this clone are transmissible among patients and they are characterized by high antibiotic multi-resistance. Our analyses showed that flagellin from B. cenocepacia K56-2 is glycosylated. To investigate the source of glycans for flagellin glycosylation, we constructed several deletion mutants in B. cenocepacia and purified flagellin from the strain K56-2 subjected to analysis by mass spectrometry and gas chromatography to define character of glycosidic bond and structure of the glycan moiety. Here we show results from our structural and genetical investigations of the glycan in the flagellin from B. cenocepacia K56-2.

SW02.S7–35
The N-terminal domain of Ultraspiracle exhibits characteristics of intrinsically disordered proteins
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Aedes aegypti is a well known mosquito vector for many serious diseases such as dengue fever or yellow fever. Development and metamorphosis of mosquito are regulated by 20-hydroxyecdysone (20E), which functional receptor acts as a transcription factor consists of two nuclear receptors: the receptor (EcR) and the Ultraspiracle protein (Usp). The N-terminal domain (NTD) of nuclear receptors is a highly variable and poorly conserved. Molecular structure, conformational changes and mechanism of function of NTD of Usp from Aedes aegypti (aaUsp-NTD), especially in the context of the full-length protein, are unknown. Preliminary bioinformatics analyses showed that aaUsp-NTD exhibits characteristics associated with Intrinsically Disordered
Proteins (IDPs) molecules. This class of proteins is very flexible. Their regions or segments possess disordered fold and they are able to adopt different conformations due to environmental conditions or binding to different partners. Due to these features IDPs play many important roles in e.g. cell cycle control, transcriptional and translational regulation, modulation of other proteins activities or regulation of nerve cell function. In our study, we characterized unusual structural state of aaUsp-NTD. To establish initial structural investigations, we established deficient, two-step purification procedure using immobilized metal ion affinity chromatography (IMAC) and gel filtration. Using size-exclusion chromatography (SEC), we investigated atypical hydrodynamic properties such as ca. 2 times larger hydrodynamic volume and ca. 1.5 times larger Stokes radius in comparison with a globular protein of the same molecular mass. Anomalous behavior was also reflected in SDS-PAGE electrophoresis. Additionally, we used sedimentation velocity ultracentrifugation (SV-AUC). Obtained results indicated that aaUsp-NTD is an asymmetric protein. Molecular mass and Stokes radius were also confirmed by SV-AUC. Secondary structure analyses, determined by the far-UV CD spectroscopy, affirmed that aaUsp-NTD is intrinsically disordered protein with residual secondary structure.

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**SW02.S7–36**

**A role of eNOS dimer stability for essential hypertension?**

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**Purpose:** Essential hypertension is associated with endothelial dysfunction and reactive oxygen species (ROS) are considered to play a role here. To investigate whether dysfunctional vascular eNOS might contribute to the regulation of blood pressure we overexpressed two different eNOS variants by the endothelial specific tie-2 promoter.

**Methods:** To accomplish this, wildtype eNOS (eNOS-tg) or respectively a destabilized eNOS (C101A-eNOS-tg) were introduced into the endothelium of C57BL/6 mice. Destabilization of eNOS was induced via replacement of a cysteine residue by alanyl resulting in impaired eNOS dimer stability, increased superoxide and decreased NO bioavailability in stably transfected HEK cells. Results

Western blot analysis proved the vascular specific overexpression of eNOS in aortic tissue of C101A-eNOS-tg (150 ± 8.4%, n = 8, p < 0.05) as well as in eNOS-tg (229 ± 15.5%, n = 6, p < 0.01) each compared to their transgene negative littermates. Destabilization in C101A-eNOS-tg resulted in elevated phosphorilation of eNOS in aortic tissue of C101A-eNOS-tg (153 ± 13.8%, n = 6, p < 0.05, versus controls). In keeping with destabilized eNOS being a source of ROS, superoxide levels were increased in C101A-eNOS-tg (223 ± 30.4%, n = 10, p < 0.01) but not in eNOS-tg (109 ± 6.3%, n = 3; myocardial tissue, both versus controls). Incubation with the NOS-inhibitor N-monomethyl-L-arginine of aortic, myocardial and skeletal muscle tissue completely inhibited the increase of superoxide. In addition, S-glutathionylation of aortic eNOS was increased only in C101A-eNOS-tg (178 ± 23.1%, n = 6, p < 0.05, versus C57BL/6) indicating augmented oxidative and nitrosative stress. Likewise, peroxynitrite levels were increased in myocardial tissue of C101A-eNOS-tg (153 ± 8.1%, n = 7, p < 0.01) but unchanged in eNOS-tg (103 ± 11.9%, n = 5) as compared to C57BL/6. Protein kinase G activity was evaluated by VASP phosphorylation at Ser239 showing no difference in both strains versus controls suggesting unchanged NO-cGMP pathway activation. Organ bath experiments revealed normal aortic reactivity to acetylcholine, phenylephrine and the NO-donor SNAP in both strains versus controls (n = 5–7). As expected systolic blood pressure measured in awake mice by tail cuff method was significantly decreased in eNOS-tg (109 ± 1.9 mmHg, n = 4, p < 0.05). In striking contrast and although dimer destabilization in C101A-eNOS-tg did reduce but not impair NO formation, blood pressure in C101A-eNOS-tg (118 ± 2.8 mmHg, n = 8) was exactly the same as compared to controls (118 ± 3.1 mmHg, n = 6). Plotting aortic endothelial eNOS expression against blood pressure using three different transgenic animal strains revealed a highly significant approximation by the equation ‘one phase exponential decay’ (R² = 0.9992). According to the expression level in C101A-eNOS-tg a blood pressure reduction of at least 4 mmHg would have been expected. Conclusion

These data demonstrate that a decrease of eNOS dimer stability (associated with increased vascular oxidative stress) likely attenuates the effect of endothelial eNOS on blood pressure. This finding might contribute to explain the pathophysiology of essential hypertension.

**SW02.S7–37**

**Molecular basis of 6-methyladenine recognition by R.DpnI restriction endonuclease**

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The 6-methyladenine (6mA) is one of the key epigenetic modifications in prokaryotes. R.DpnI is the best known 6mA-dependent restriction endonuclease, specific for Dam methylated G6 mATC sites and widely used for site-directed mutagenesis.

Our previous studies have shown that R.DpnI consists of two domains, an N-terminal catalytic PD-(D/E)XXK domain and a C-terminal winged helix (WH) domain and that both independently read out DNA sequence and methylation status. In our first structure of R.DpnI-DNA complex, there is only one substrate oligoduplex per enzyme molecule, which is specifically bound to the WH domain and distant from the catalytic domain [1]. Hence, the question remained open how the catalytic domain of R.DpnI interacts with its target, and how it specifically recognizes the methyl groups which license DNA cleavage. Such a process is difficult to realize with stringency, because attractive van der Waals interactions with the small hydrophobic group are relatively weak when compared to repulsive ones. The structures depicting the phenomenon are rare and there is still relatively little known about the mechanism of recognition by modification dependent enzymes.

Here, we present a high resolution structure of R.DpnI which features both protein domains bound to the target DNA. Recognition of the 6mAs by the catalytic domain is carried out without flipping bases out of the helix. A previously disordered loop wraps around the major groove of the target DNA, where both methyl groups are located in close proximity to each other. The PD-(D/E)XXK domain places the 6mAs in a hydrophobic pocket.
formed by residues Leu129 and Trp138. To the best of our knowledge this is the first structure showing how a protein can efficiently detect two 6mA modified sites together.

**Reference**

**SW02.S7–38**
DNA binding, allosteric regulation and PipX coactivation clarified structurally for the NtcA global nitrogen regulator of cyanobacteria
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Transcription regulators of the Crp-Fnr superfamily are key players for bacterial gene expression regulation. Among them, NtcA orchestrates in cyanobacteria the gene expression response to nitrogen starvation, commanding a large regulon, being activated by 2 exoglutamate (2OG, a nitrogen starvation signaling molecule) and coactivated by protein PipX. We illustrate the constancy of the regulator-DNA complex structure within the Crp-Fnr superfamily by determining crystal structures of NtcA, bound to its target DNA sequence. In addition, we provide with NtcA a unique glimpse of the architecture of a Crp-Fnr-family factor being bound simultaneously to target DNA, small molecule activator (2OG) and protein modulator (PipX), by determining the corresponding crystal structure for this quaternary complex. We also prove that the structures of 2OG-activated NtcA alone or bound to DNA are identical except for changes in the conformations of side chains of DNA-interacting residues. PipX links NtcA regulation with nitrogen control mediated by the signaling protein PII. Under conditions of nitrogen abundance, PII sequesters PipX, rendering this coactivator unavailable for NtcA activation. Our structure of NtcA bound to both 2OG and PipX led us to conclude that PipX coactivates NtcA by stabilizing the active conformation of NtcA. However, it was very important to ascertain whether PipX mediates in part its coactivation role by interacting with DNA. The present structures of the DNA-NtcA and PipX-NtcA-DNA-complexes exclude such interaction of PipX with the DNA, which is highly bent (approximately 80°) by the transcription factor. These structures fully clarify the mechanism for the exquisite specificity of NtcA for its target DNA sequence, for which we provide mutational evidence. Such specificity is essential for discrimination between the NtcA box and the highly similar Crp box, since Crp and NtcA coexist in cyanobacteria, and each one of them controls a different regulon. Projects BFU2011-30407 of MEC Spain, and Prometeo of Generalitat Valenciana

**SW02.S7–39**
Recognition of the methionylated initiator tRNA by the translation initiation factor 2 in Archaea
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Heterotrimeric αIF2αβγ (archaeal homologue of the eukaryotic translation initiation factor 2) in its GTP-bound form delivers Met-tRNA^Met to the small ribosomal subunit. After recognition of the start codon, this factor in its GDP-bound form loses affinity for tRNA; and subsequently dissociates from the initiation complex. It is known that the heterodimer containing the GTP-bound γ subunit and domain 3 of the α subunit of αIF2 is necessary and sufficient for the formation of a stable ternary complex with Met-tRNA^Met. Such heterodimer in complex with GDPNP and Met-tRNA^Met was crystallized the ternary complex structure has been solved.

In contrast to the structure of the elongation ternary complex EF-Tu-GDPNP-Phe-tRNA^Phe, in the presented structure of the initiation ternary complex Met-tRNA^Met contacts the protein through the acceptor stem, the D stem-loop and the CCA-Met end, which is positioned between the G domain and domain III of the αIF2 γ subunit. All three domains of the γ subunit and domain 3 of the α subunit contact Met-tRNA^Met. The overall shape and domain arrangement of the γ subunit in this complex differ from that described previously: domains II and III shifted relative domain I and as a result free space forms between domains I and III. The presented structure of the initiation ternary complex is in good agreement with biochemical and hydroxyl radical probing data. Remarkably, the recently published 5.0 Å resolution structure of almost the same ternary initiation complex differs dramatically from the structure presented. Reasons for this discrepancy are discussed.

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**SW02.S7–40**
Asn2 mutations in alphaIIb integrin lead to a structural deformation of a calcium-binding site and a defective expression of alphaIIbbeta3 complex
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The platelet integrin αIIbβ3 serves as a structural and a functional model representing the integrin protein family. Recently we discovered a new Asn2Asp substitution in the β-propeller domain of the αIIb subunit, leading to a Glanzmann thrombocytopenia bleeding disorder. Alignment with other integrin α subunits reveals that Asn2 is highly conserved. Although the mutant αIIbβ3 complex is formed, the mutation impairs its intracellular trafficking from the ER to the Golgi. Substitution of Asn2 to uncharged Gin only partially decreases αIIbβ3 surface expression. The αIIb subunit contains four calcium-binding sites which are important for αIIb biogenesis and stability. Molecular dynamics simulations were used to explore the structural impact of these substitutions. We show that the natural Asn2Asp mutation totally disrupts a hydrogen bond between Asn2 and Leu366, whereas an intermittent contact is evident in an artificial Asn2Gln mutant. Leu366 is a non-coordinating residue of a calcium binding site 3 in the β-propeller. Disruption of a hydrogen bond between Asn2 and Leu366 affects the structure of a calcium-binding site, causing its expansion. The level of this structural deformation in a binding site is correlated to the level of αIIbβ3 surface expression. A comparison to other calcium-binding sites of αIIb and of other α integrins indicates conservation in the size of a loop containing calcium coordination residues. The simulation data is further verified by EDTA chelation assay, in which, sensitivity to calcium removal corresponds with the calcium binding site expression and with the reduction in surface expression. Our findings indicate that Asn2 mutations cause a structural deformation in a calcium-binding site 3 of αIIb, which is proposed to affect a binding of a calcium ion and consequently an integrin intracellular trafficking and a surface expression. We suggest that the ability of αIIb to bind calcium relies on a strictly defined structure of a calcium-binding site.
Purification and characterization of novel laccase from basidiomycete Antrodia faginea 1998

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Laccases belong to the family of blue multicopper oxidases. They oxidize a wide range of phenolic and some non-phenolic compounds, performing one-electron reduction of the molecular oxygen at the active center. The enzymes active center contains four copper ions that are classified as type 1 (T1), type 2 (T2) and type 3 (T3). The copper ion of type 2 and two copper ions type 3 forms T2/T3 trinuclear cluster.

Laccases might be used in the several fields including food, pulp and paper, textile, cosmetics industry and another. The key point in biotechnological application of laccases is their catalytic efficiency. It is well documented that the catalytic efficiency depends on redox potential of T1 copper. Laccases have been classified as low-, medium- and high-redox potential enzymes based on equilibrium potentiometric titrations of T1 site. Fungal laccases, especially from ligninolytic basidiomycetes, have higher redox-potentials for the copper sites compared with those from other organisms.

The new laccase from white-rot basidiomycete Antrodia faginea 1998 have been studied. Two genes encoding laccases have been revealed in the mycelial fungus A. faginea: lac1 and lac2. The laccase preparation was purified to homogeneity according to protocol developed. The MALDI-TOF-TOF mass spectrometry analysis of laccase isoenzymes allowed identifying a major isoenzyme as expression product of lac2 gene. The molecular mass of Lac2 was 65 kDa and the isoelectric point – 4.8. The spectroscopic characteristics of Lac2 were typical for classic ‘blue’ laccases. The Lac2 substrate specificity was examined using various phenolic and non-phenolic substrates. The Lac2 thermo optimum was quit low in comparison with those for other fungal laccases. Decolorisation test showed that the redox-potential of laccase is lower than the redox potential of Trametes maxima laccase (750 mV), because it doesn’t fully decolorize Azure B with HBT, that allows to attribute A. faginea laccase to medium-redox potential laccases.

The 3D structure of Lac2 was solved at 1.75 Å resolution. The comparative structural analysis of the Lac2 and high-redox potential fungal laccases revealed significant differences in substrate-binding pocket organization.

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Intracellular laccase of basidiomycete Trametes hirsuta

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Laccases (EC 1.10.3.2) are copper-containing phenol oxidases catalyzing oxidation of a broad range of aromatic substrates including o,p-diphenols, aminophenols, polyphenols, polyamines, model lignin structures and some inorganic compounds. There is a wealth of information about their reaction mechanism, biological role and molecular organization, nevertheless the process of laccase biosynthesis still remains unclear. Laccases synthesis and secretion are influenced by nutrients levels, culture conditions, developmental stage and presence of different inducers.

The aim of this work was to clarify the stages of laccase biosynthesis in the white-rot fungus Trametes hirsuta. For this purpose the pool of intracellular laccases was isolated from mycelia extracts that was harvested after fungus cultivation on glucose-peptone medium supplied with CuSO4 - an inducer of enzyme biosynthesis. Using the developed separation procedure we collected two fractions of homogenous intracellular proteins with high level of production within the cell. Western-blot analysis with Trametes hirsuta native laccase antibodies confirmed that these homogenous intracellular proteins were laccases. Both of them have shown to possess the high specific laccase activity (LacA = 471.2 U/ml, LacB = 239.0 U/ml), SDS-PAGE analysis showed that purified laccases were monomeric proteins of approximately 64.0 kDa. The laccases isolated had different pIs (pI A = 3.8, pI B = 3.3) while their Mr remained almost the same. Mass-spectrometry analysis has revealed tryptic digestion spectra typical for laccases but showed differences in level of glycosylation of two laccase forms. Alignment of peptide sequences derived after MALDI-TOF/TOF analysis of fragmentation spectra revealed that both proteins refer to the product of the same gene encoded major laccase isoform. Currently three laccase
genes with close expression level have been sequenced in T. hirsuta transcriptome. However the laccases encoded by two other genes have not been identified both in cell extract and cultural broth. Results of this study provide new insight to the mechanism of laccase synthesis, folding and secretion in Basidiomycete.

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SW02.S7–44
Identification a stable monomeric form of K315A mutant delta-crystallin on the pathway of unfolding
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Delta-Crystallin is a taxon specific protein in eye lens of birds and reptilia. It is a homotetramer with three domains. Our previous study found out that K315 in double dimer interface are elusive. Previously, it was inferred that K315A mutant of delta-crystallin on the pathway of unfolding.

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SW02.S7–45
The critical role of cis-trans Pro32 isomerization in misfolding and aggregation of human beta2-microglobulin explored by chemical protein synthesis
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Human beta2-microglobulin (b2m) deposits as amyloid fibrils within joints during long-term hemodialysis treatment. Despite the devastating effects of dialysis-related amyloidosis, a full understanding of how fibrils form from soluble b2m remains elusive. Previously, it was inferred that cis-to-trans amide bond isomerization of the cis-Pro32 residue triggers a transition of the native form of b2m into an aggregation-prone conformer. We used chemical synthesis to prepare a series of b2m analogues in which Pro32 residue was systematically substituted with several nonstandard amino acids, including 4-(S)-fluoroproline, 4-(R)-fluoroproline, 4-methyl-proline (Mpr), which have different propen-
sities to adopt either a cis or trans conformation of the prolyl amide bond. We find that such substitutions indeed affect cis-to-trans populations of conformers when inserted into the b2m polypeptide chain, and the resulting molecules exhibit different protein fold stabilities. The most ‘trans-conformation favoring’ Mpr32 analogue was found to display particularly interesting properties: lowest stability of protein fold, spontaneous aggrega-
tion into oligomers at neutral pH, affinity to Rifampicin SV concomitant with a change in oligomerization pathway. Strikingly, it also showed lowest kinetics of amyloid growth in a ‘seed extension assay’ at pH 2.5 in contradiction to the hypothesis that trans-Pro32-conformer is a precursor to the amyloids formation. Currently, we are performing studies on other chemically synthesized analogues (4,4′-difluoroproline, azetidine-2-carboxylic acid, beta3-proline, and α,α′-aminoisobutyric acid) in order to test the alternative hypothesis that local flexibility at site 32 is required for efficient growth of amyloid fibrils.
SW02.S7–47
How to determine the size of the nucleus of protofibrils from the concentration dependence of the lag-time of aggregation? Experimental application: insulin and Lys-Pro insulin

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The question about the size of nuclei of protofibrils formed by different proteins and peptides is yet open. By the nucleation mechanism, the formation of protofibrils begins from the thermo-dynamic unfavorable steps resulting in the formation of a critical nucleus consisting of n monomers. The kinetic model of the process of formation of amyloid fibrils is suggested in our work allowing us to determine the size of the nucleus using kinetic data. In addition to the stage of nucleation, the given model includes both a linear growth of protofibrils (proceeding only at the cost of attaching of monomers to the ends) and an exponential growth of protofibrils at the cost of branching and fragmentation. Theoretically, only the exponential growth is compatible with the existence of a lag-period in the fibril formation kinetics. Insulin is a commonly used protein for studies of amyloidogenesis. There are a few insulin analogues with different pharmacokinetic characteristics, in particular the onset and duration of action. As the duration of action may be connected with the duration of the lag-phase, the challenge is to consider the process of amyloid formation for different analogs of insulin. One of them is LysPro insulin. The behavior of LysPro insulin in the process of amyloid formation has not been studied in detail yet. To quantitatively investigate the differences between the two samples in the aggregation reaction and estimate the difference in the lag-time, we used thioflavin T fluorescence assay, electron microscopy, X-ray diffraction methods, and theoretical modeling. Kinetic experimental data for both insulin and LysPro insulin samples demonstrated the increasing of the lag-time for LysPro insulin at low concentrations of monomers, particularly at 2 and 4 mg/ml, which corresponds to the pharmaceutical concentration. The obtained analytical solution and computer modeling allowed us to determine the size of the nucleus from the experimentally obtained concentration dependences of the relationship between the lag-time and the time of growth of amyloid fibrils. In the case of both insulin and LysPro insulin, this relationship is independent of the protein concentration. According to the developed theory, this means that the size of the nucleus corresponds to one monomer in both insulin and LysPro insulin.

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SW02.S7–48
The type 2B p.R1306W natural mutation of von Willebrand factor (VWF) dramatically enhances the multimer sensitivity to shear stress

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Shear stress triggers conformational changes of von Willebrand factor (VWF), responsible for both binding to the platelet receptor GpIbα and its self-association that support primary haemostasis under flow. Type 2B VWF mutants are considered to have an increased affinity for platelet GpIbα. We performed surface plasmon resonance measurements on the interaction of GpIbα with WT and p.R1306W VWF multimers as well as A1-A2-A3 constructs. Full length WT-VWF does not interact with GpIbα under static conditions, whereas the mutant at 2 μg/ml already showed a significant interaction with the receptor. By contrast, the WT and p.R1306W A1-A2-A3 constructs showed comparable affinities for GpIbα (KD = 20 nM). The analysis of VWF multimers self-association was performed by atomic force microscopy (AFM) and dynamic light scattering (DLS) spectroscopy over a 0–60 dyn/cm² shear stress range. At shear stress <10 dyn/cm², the p.R1306W multimers already unfold and undergo an initial self-aggregation with formation of a three-dimensional network having increased roughness compared to WT-VWF. DLS showed that at rest the hydrodynamic radius of WT-VWF multimers was significantly lower than that of the R1306W mutant (87 ± 22 versus 210 ± 60 nm). Mechanical stretching AFM experiments showed that p.R1306W multimers needs less energy per length unit (≈10pN) to be stretched compared to WT counterpart. Thus, the increased avidity for platelet receptors of p.R1306W VWF arises mostly from an increased sensitivity to shear stress that facilitates exposure of GpIbα binding sites and proteolytic attack by ADAMTS-13.

SW02.S7–49
Intracellular localization of recombinant human cardiac troponin I in the mammalian cell culture

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Troponin I is a protein that is included in the troponin complex along with troponin T and troponin C. Troponin I is involved in the regulation of the muscular cell contraction. Recent data indicated TnI localization in the nuclei of cardiomyocytes. It was suggested that nuclear TnI maintains the integrity of the chromosomes and regulates the gene expression.

The aim of the study was to investigate distribution of recombinant human cardiac troponin I being expressed in different types of cells: myoblast cell line as well as in cells of non-muscle origin. Gene sequence corresponding human cardiac troponin I (hTnI) was cloned into the plasmid pcDNA6mycHisA for the
subsequent expression in the eukaryotic cells: HEK, CHO, HeLa and c2c12.

Detecting of the expression and localization of the hcTnI were performed in different ways. Cells were fixed with 4% paraformaldehyde and troponin localization was revealed with specific fluorescently labeled anti-hcTnI antibodies. Additionally transfected cells were fractionated into nuclear and cytoplasmic fractions followed by hcTnI quantitative measurement utilizing a pair of specific anti-hcTnI antibodies (sandwich-type fluorimunoassay method).

During the expression of the hcTnI in the non-differentiated c2c12 and non-muscle cells (HeLa, HEK, CHO) the protein was detected in the nucleus after antibody staining with the specific antibodies. Sandwich-type immunassay analysis detected the protein in the nuclear fraction of cell lysates whereas its level in cytoplasmic fraction was undetectable. Being expressed in the differentiating myoblasts hcTnI changed localization: the protein was associated with filament structures as it was shown by staining cells with anti-hcTnI antibodies.

Using the PSORT II program we found several nuclear import signals in the primary structure of TnI. Embedding of the hcTnI in mouse troponin complex was confirmed by recombinant assembly complex in vitro.

Conclusion: (i) being expressed in non-differentiated cells of muscle origin and in non-muscle cells TnI localizes in the nucleus; (ii) expression of recombinant hcTnI in differentiating muscle cells leads to protein association with forming contractile apparatus; (iii) TnI of human cardiac origin is able to be included in mouse contractile system.

SW02.S7–50
Telomerase protein Est3 from H. polymorpha
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Telomeres are essential for genome stability in all eukaryotes. Changes in telomere functions and the associated chromosomal abnormalities have been implicated in human aging and cancer. Telomerase – is a multisubunit enzyme that provides genome stability by elongation of ends of the telomeres in the immortal cells (in particular germ, stem and cancer cells).

Est3 – is a small regulatory subunit of yeast telomerase which is dispensable for enzyme catalysis but essential for telomere replication in vivo yeast Saccharomyces cerevisiae.

We show here an identification of Est3 protein from the methylotrophic yeast Hansenula polymorpha (syn. Pichia angusta). Deletion of hpEst3 gene leads to senescence phenotype and telomere shortening. We created a construction, which permits to obtain a protein with removable tag. The hpEst3 protein was isolated, cleaved and purified for further structural analysis.

After several primary crystallization screens we observed the data about initial crystallization conditions.

Availability of an atomic structure of the telomerase components will allow elucidation of in vivo mechanisms of telomerase action and open new possibilities for the development of novel agents for the normalization of cell division in cancer cells.

SW02.S7–51
DNA methyltransferase SsoII: a balance between DNA methylation and transcription repression
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Restriction-modification (R-M) systems serve as primitive immune systems protecting bacterial cells from phage infection. R–M system SsoII from Shigella sonnei consists of a DNA methyltransferase (M.SsoII) and a restriction endonuclease (R.SsoII). M.SsoII methylates C5 atom of the second cytosine in the sequence 5′-CNGG-3′ (N = A, C, G or T) in dsDNA while R.SsoII cleaves this site in case it remains unmethylated. Since phage DNA is not methylated, R.SsoII hydrolyzes it and protects the host cell. In case the endonuclease activity exceeds the methyltransferase activity, the host cell DNA can be cleaved as well. Therefore, M.SsoII and R.SsoII expression should be strictly coordinated.

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SW02.S7–52
SeqOPT: web based server for rational design of conformationally stable alpha-helices in monomeric peptides and globular proteins
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Rational design of peptide’s and protein’s helices is not only of practical importance but also is important for better understanding of protein folding. Recent theoretical models of helix-coil transitions in monomeric peptides allow accurate predictions of their helical stability. This provides a good basis for design of peptides and proteins with increased stability. We present a web-based tool (available at http://mml.spbstu.ru/seqopt) for the de novo design of highly stable α-helices using SeqOPT algorithm.


SW02.S7–53

The domains of \textit{Staphylococcus aureus} haemoglobin receptor, IsdH, cooperate to steal haem from human haemoglobin

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The nutrient iron is essential for bacterial growth and infection. Pathogenic bacteria have evolved multiple mechanisms for acquiring iron from the infected host. The iron regulated surface determinant (Isd) pathway of \textit{Staphylococcus aureus} is highly up-regulated during infection and targets the largest mammalian pool of iron, the haem protein haemoglobin A (Hb). The Isd system contains nine proteins that capture Hb on the cell surface, liberate the haem cofactor and relay it through the cell wall and membrane into the cytoplasm where it is degraded, releasing iron. Two related proteins IsdB and IsdH are expressed on the bacterial cell-surface where they act as receptors for HbA and extract the haem molecule from the host protein. Both Hb and the isolated haem ligand are bound by near iron-transport (NEAT) domains, which are found in multiple copies in IsdB and IsdH. It is the combination of NEAT domains in the full-length proteins that allows for rapid haem removal from Hb as the isolated NEAT domains do not retain this function. We have determined the structure of the intact conserved region of IsdH bound to Hb and shown that no large changes in the globin structure occur. Instead the haem binding NEAT domains of IsdH make direct contacts with the haem pockets of Hb. IsdH is positioned to access all four haem groups carried by one Hb molecule and functional studies confirm this activity. Despite the high sequence similarity of IsdH and IsdB, we have shown functional differences in their mechanism of Hb capture and haem transfer activity. This provides the first insight into how the domains of these receptors cooperate to achieve rapid haem transfer out of the captured globin molecule.

SW02.S7–54

X-ray crystallographic study of VapD from the phytopathogen \textit{Xylella fastidiosa}: implications for DNA binding

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Virulence-associated proteins (Vaps) have been found in many organisms, but as yet little is known about their structure and function. There is an evidence suggesting that Vaps should be related to CAS2 family of ribonucleases associated with the CRISPR system of microbial immunity. Here we report a crystal structure of the 16.3 kDa VapD protein from the phytopathogen \textit{Xylella fastidiosa}. Crystals were grown by the hanging drop method by mixing 1.8 \( \mu \)l of a protein solution (9.8 mg/ml) with an equal amount of 20% (v/v) MPD and 0.1 M glycine in 0.1 M NaOAc, pH 4.5. Diffraction data were collected at a wavelength of 1.46 A using MAR Mosaic CCD 225 detector on the W01BMX2 beamline of the Brazilian Synchrotron Light Laboratory. Crystals were found to be immanent merohedral twins with a twinning fraction of 0.48. In spite of the lack of suitable sequence homology, a successful solution was found by the molecular replacement method with a search model prepared ab initio using the ROSETTA suite. The refined protein model confirms the accuracy and reliability of the solution found, with R-factor and R-free values equal to 0.250 and 0.308, respectively. In order to elucidate a possible mechanism of action, a search for structural analogous was carried out in PDB. Structural alignment using DALI revealed a probable DNA binding cleft on the surface of the protein. Computer molecular dynamics simulation with GROMACS has proved a stability of the suggested DNA-protein complex. The refined model furnished structural details which may provide an important evidence for the VapD function.

SW02.S7–55

Experimental determination of the formation sequence of structure elements in the green fluorescent protein

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The most complex problem in studying multi-state protein folding is the determination of the sequence of formation of protein intermediate states. A far more complex issue is to determine at what stages of protein folding its various parts (secondary structure elements) develop. The structure and properties of different intermediate states depend in particular on these parts. An experimental approach, named m-analysis, which allows understanding the sequence of formation of structural elements upon folding of a multi-state protein was used in this study [Melnik T.N. et al., PLoS One, 2012, 7(11):e48604]. In this approach, the same
elements of the protein secondary structure are ‘tested’ by substitutions of single hydrophobic amino acids and by incorporation of cysteine bridges. Single substitutions of hydrophobic amino acids contribute to yielding information on the late stages of protein folding while incorporation of ss-bridges allows obtaining data on the initial stages of folding.

In the present work we have investigated GFP-cycle3 upon folding of which at least two intermediate states formed. Having analyzed mutant forms of this protein, we tried to understand the formation sequence of its structure elements. GFP-cycle3 seems to be the most appropriate protein for such studies. First, the chromophore formation and fluorescence of mutant forms of this protein evidences unambiguously that the mutation did not disturb the general protein structure. Second, the structure of GFP-cycle3 represents a b-can and allows designing almost at any place not only single amino acid substitutions but also ss-bridges between neighboring beta-strands.

Our microcalorimetric analyses of the mutant forms of this protein when its melting is a non-equilibrium process allowed us not only calculate the rate constants of the GFP-cycle3 unfolding but also obtain data on the effect of these mutations on the entropic and enthalpic components of the protein energy barriers.

In turn, this permitted us to establish the formation sequence of beta-hairpins upon the GFP-cycle3 folding.

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SW02.S7–56
Substrate binding to 2-aminobenzoyl-CoA monoxygenase/reductase from Azoarcus evansi

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2-Aminobenzoic acid (anthranilic acid) is an important intermediate in the synthesis and degradation of many N-heterocyclic compounds such as tryptophan. As a consequence of its wide occurrence, 2-aminobenzoate is a common substrate for many microorganisms that are able to cleave aromatic rings. In Azoarcus evansi the initial degradation of 2-aminobenzoate to a nonaromatic product starts with the 2-aminobenzoate-CoA ligase and 2-Aminobenzoyl-CoA monoxygenase/reductase (ACMR).

ACMR is a bifunctional flavoenzyme which catalyzes both monoxygenation and hydrogenation of 2-aminobenzoyl-CoA requiring 2 NADH and one O2. The reaction can be divided in an initial hydroxylation catalyzed by the monoxygenase domain and subsequent reduction of the intermediate to a nonaromatic product.

Characterization of the recombinantly expressed ACMR revealed that the homodimeric protein contains one FAD (in the monoxygenase domain) and one FMN (in the reductase domain) as redox cofactors.

Protein modeling of the two subdomains indicates that the reductase domain shows high similarity to proteins from the OYE family. Based on difference titration experiments we suggest that the active site of the enzyme has two separate binding sites to which the corresponding substrate or substrate analog (4-hydroxybenzaldehyde) can bind simultaneously.

Furthermore, ACMR was characterized by circular dichroism, small angle X-ray scattering and ThermoFAD experiments. The protein shows a typical CD spectrum for a mixed α-helix/β-sheet protein and a Tm of 45°C. Protein stability in different buffers was monitored using the ThermoFAD method. ACMR showed only one transition and is completely or partially unfolded at pH-values lower than 5.5.

SAXS measurement confirmed that ACMR is a homodimer in solution with a calculated molecular mass of ca. 165 kDa. A representative low-resolution ab initio model showing the shape of ACMR was calculated from the SAXS data.

SW02.S7–57
Interaction between linker histone H1 and non-histone protein HMGB1 in vitro

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The linker histone H1 is one of the major architectural proteins at the nucleosomal and post-nucleosomal levels of chromatin structural organization. The histon H1 binds to DNA at the entrance/exit of the nucleosome core particle and plays an important role in maintaining of higher order chromatin structures. However, other proteins such as members of the HMG superfamily also participate in maintenance of the chromatin structure. For example HMGB proteins interact with linker DNA having their binding sites in the close proximity to those of H1 that might lead to the interaction between the proteins.

Using electrophoretic and spectroscopic approaches we have studied the interaction between the H1 and HMGB1 proteins in solution. We have shown that the H1 and HMGB1 tend to form multi molecular complexes. Circular dichroism data indicate that detectable changes in the secondary structure of at least one of the proteins in the HMGB1/H1 complex have been observed.

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SW02.S7–58
Random approach to stabilize a membrane transport protein for crystallization studies

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X-ray crystallography is one of the most powerful techniques to study proteins at the atomic level. Unfortunately, obtaining high quality crystals of membrane proteins is a difficult task due to the hydrophobic nature of these proteins. The low stability in solution of these proteins is the biggest problem during crystallization studies. One of the most common strategies to overcome these problems consist in working with functional mutants of these proteins. Despite this, predicting what mutations are going to improve the stabilization of a protein is virtually impossible. We have built up a medium-high throughput experimental protocol with objective to generate and characterize random mutants of a membrane protein with more stability in solution and, therefore with a better probability to crystallize. The combination of random mutagenesis with rapid and sensitive screening protocols
of protein expression and stability seems to be the best approach for this goal. We optimized an assay based on a split GFP to build and characterize the random mutants library of SteT (Bacillus subtilis transporter that exchanges L-threonine by L-serine). SteT is an excellent prokaryotic model of the mammalian L-amino acid transporter (LAT) family. Genetic mutations of some LATS are the direct cause of two types of aminooacidurias. Also, a member of this family, LAT1, is overexpressed in tumor cells. Unfortunately, SteT wild type solubility and stability in detergent solutions is very low and completely incompatible with crystallization tests. Our results suggest that random mutagenesis combined with the detergent split assay, appears to be an excellent strategy to build robustness in membrane proteins for structural studies. So far, using this strategy we found a mutant of SteT that currently is undergoing for crystallization screenings to study the structure and mechanism of mammalian LATS.

**SW02.S7–59**

**Supramolecular structures formed by TIP49A protein in vitro**

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Tip49a, a human protein isolated with several chromatin-remodeling complexes, is readily oligomerized in vitro forming poly-disperse aggregates of the size of tens and hundreds of nanometers. In this work we show that the non-specific aggregation of the protein can be effectively countered by 0.05–0.1% concentrations of a detergent Triton-X100. We also show that addition of the detergent destroys already formed aggregates, that allows us to isolate oligomeric forms of the protein that may have biological significance. When combined, the results of small angle X-ray scattering and dynamic light scattering experiments suggest that TIP49A aggregation observed in vitro is reversible with the protein oligomerization in two different types of stable filamentous structures.

**SW02.S7–60**

**Contributions to the study on the interactions between blood proteins and monolayers and/or liposomes**

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We report our contributions to the study on the interactions between the blood proteins human serum albumin (HSA) and human immunoglobulin G (IgG) with monolayers and/or liposomes formed by DMPC, DPPC and F-DPPC. Dynamic light scattering, flow cytometry, enzyme-linked immunosorbent assay (ELISA), electrophoretic mobility, differential scanning calorimetry (DSC), surface tension measurements, surface film balance and Brewster angle microscopy (BAM) have been used. HSA and IgG interact with liposomes forming aggregates that remain stable at protein concentrations beyond those of total liposome coverage. Both HSA and IgG penetrate into liposome bilayer. An ELISA assay indicates that the F₄ region of IgG is the one that is immersed in the DMPC membrane. The liposome-protein interaction is mainly of electrostatic nature. HSA/DPPC mixed monolayers exhibit two phase transitions evidenced by two discontinuities in the corresponding isotherms and two minimum values in the compressional modulus-surface pressure curves. Relative thickness values of HSA/DPPC mixed monolayers showed the existence of an exclusion surface pressure below which the monolayer is composed of both components. BAM images of HSA/F-DPPC system reveal that some protein molecules in a packed ‘loops’ configuration remain at the interface at surface pressures higher than the ‘exclusion’ surface pressure.

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**References**


**SW02.S7–61**

**Structural bioinformatics of the human spliceosome**

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The spliceosome is one of the largest molecular machines known. It performs the excision of introns from eukaryotic pre-mRNAs. In human cells it comprises five RNAs, over one hundred ‘core’ proteins and more than one hundred additional associated proteins. The details of the spliceosome mechanism of action are unclear, because only a small fraction of spliceosomal proteins have been characterized structurally in high resolution. To aid structural and functional analyses of the spliceosomal proteins and complexes, and to provide a starting point for multiscale modeling, we carried out a comprehensive structural bioinformatics analysis of the entire spliceosomal proteome.

We discovered that almost a half of the combined sequence of proteins abundant in the spliceosome is predicted to be intrinsically disordered, at least when the individual proteins are considered in isolation. The distribution of intrinsic order and disorder throughout the spliceosome is uneven, and is related to the various functions performed by the intrinsic disorder of the spliceosomal proteins in the complex. In particular, proteins involved in the secondary functions of the spliceosome, such as mRNA recognition, intron/exon definition and spliceosomal assembly and dynamics, are more disordered than proteins directly involved in assisting splicing catalysis. Conserved disordered regions in splicing proteins are evolutionarily younger and less widespread than ordered domains of essential splicing proteins at the core of the spliceosome, suggesting that disordered regions were added to a preexistent ordered functional core. The spliceosomal proteome contains a much higher amount of intrinsic disorder predicted to lack secondary structure than the proteome of the ribosome, another large RNP machine. This result agrees with the currently recognized different functions of proteins in these two complexes.

For the ordered part of the spliceosomal proteome, we carried out protein structure prediction. We identified new domains in spliceosomal proteins and predicted 3D folds for many previously known domains. We also established a non-redundant set of experimental models of spliceosomal proteins, as well as
constructed in silico models for regions without an experimental structure. Altogether, over 90% of the ordered regions of the spliceosomal proteome can be represented structurally with a high degree of confidence. The combined set of structural models for the entire spliceosomal proteome is available for download from the SpliProt3D database (http://iimcb.genesilico.pl/SpliProt3D). Finally, we analyzed the reduced spliceosomal proteome of the intron-poor organism *Giardia lamblia*, and as a result, we proposed a candidate set of ordered structural regions necessary for a functional spliceosome.

References

**SW02.S7–62**

Interaction between polyamidoamine dendrimers and regulatory plasma proteins: alkaline phosphatase and L-lactic dehydrogenase

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Dendrimers are relatively new organic polymers topologically based on the structure of the trees. They possess many functional end groups, which are responsible for high solubility and reactivity, and have empty internal cavities. These properties make them suitable for targeting, microarray systems, catalysis or drug delivery systems, carriers of genetic material or contrast agents in clinical imaging. However, due to the exogenous nature of these compounds before they are introduced into the body, it is necessary to examine their interactions with regulatory plasma proteins. Regulatory plasma proteins which constitute <1% of plasma proteins are proteins, such as enzymes, proenzymes and hormones.

The aim of the present study was to investigate interaction between two polyamidoamine (PAMAM) dendrimers (generations 3 and 4) and regulatory plasma proteins. We have chosen two of these proteins: alkaline phosphatase and L-lactic dehydrogenase to study the interaction with dendrimers. Alkaline phosphatase are responsible for removing phosphate groups from many types of molecules, while, L-lactic dehydrogenase is an enzyme which catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. It converts pyruvate, the final product of glycolysis, to lactate when oxygen is absent or in short supply, and it performs the reverse reaction during the Cori cycle in the liver.

The interactions between dendrimers and proteins were examined by zeta potential measurements and dynamic light scattering. Based on the obtained results the molar ratios of protein-dendrimer complex were calculated. Furthermore, the effect of dendrimers on protein secondary structure was studied using circular dichroism spectroscopy and their effect on protein molecular dynamics was analysed by room temperature tryptophan phosphorescence. It was shown, that both secondary structure of proteins and the molecular mobility of protein structures were changed as a result of its interaction with dendrimers. This fact reflects binding of dendrimers to the protein molecules.

The results obtained contribute to the understanding of the mechanisms of PAMAM dendrimer/regulatory plasma membranes interactions.

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**SW02.S7–63**

Application of mass spectrometry to the characterization of post-translational modifications of chromosomal proteins

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Linker histone H1 and non-histone chromosomal protein HMGB1 are some of the most abundant chromatin proteins. Both proteins play an important role in the structural organization of chromatin at the post-nucleosomal level. Post-translational modifications such as acetylation, methylation, and phosphorylation are considered to be regulators of functioning of these proteins in the chromatin. We have studied the post-translational modifications of H1 and HMGB1 using mass spectrometry.

The proteins were isolated from calf thymus by extraction with perchloric acid. The fragmentation of the protein molecules was achieved using two alternative approaches. Some samples were digested with trypsin, while the others were fragmented by helium ions.

The mass spectra of proteins H1 and HMGB1 were analyzed using Mascot and ProteinProspector software. We performed a comparative analysis of the results obtained by the different experimental approaches. The advantages and disadvantages of each approach is discussed.

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**SW02.S7–64**

How calcium and Bmh1 activate yeast neutral trehalase Nth1?

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Yeast neutral trehalase (Nth1, EC 3.2.1.28) is a highly conserved enzyme which was found in many organisms. Nth1 from the *Saccharomyces cerevisiae* hydrolyses the cytosolic disaccharide trehalose into two molecules of glucose. The activity of Nth1 is regulated by PKA protein phosphorylation, yeast 14-3-3 (Bmh) protein binding and by calcium [1]. Specific EF-like motif D14TDKNVQITIED125 is located in the N-terminus of Nth1. This motif is conserved in many calcium binding proteins. Resi-
due D114 and D125 are probably responsible for calcium binding and I121 is important for a correct conformation of the motif [2]. Therefore we prepared four Nth1 mutants with one-point mutation in this motif. For our study we used analytical ultracentrifugation, enzyme-kinetic measurements and hydrogen/deuterium exchange coupled to mass spectrometry (HDX-MS) to reveal how the calcium and Bmh1-binding affect the activity of Nth1. Our kinetic measurements revealed that Nth1 mutants D114 and D125 are inactive and that the Bmh1-dependent activation of Nth1 is significantly more potent than the calcium dependent one. From the HDX-MS measurements we suggest that regions surrounding the buried active site of pNth1 directly interact with Bmh1. These regions undergo a structural change and thus enable easier substrate and products entry and departure. The calcium dependent structural changes of Nth1 revealed that region containing putative calcium binding site and segments from the vicinity undergo a significant structural change in the presence of calcium. This might reflect an interaction between the catalytic domain and upstream located calcium binding domain and suggest an explanation for the calcium dependent activation of Nth1. Sedimentation velocity measurement was used to check the oligomeric status of the Nth1 mutants.

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References


SW02.S7–66

Structure of RecX complex with the presynaptic RecA filament: molecular dynamics simulations and small angle neutron scattering

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RecX, a small bacterial protein, is known as a negative regulator of the homologous recombinase RecA which is the essential part of the DNA reparation machinery in bacteria. RecX has been shown to bind to RecA filament and inhibit its activity of the homologous strand exchange. An ability of RecX to form a filament by binding to the grove on RecA:ssDNA presynaptic complex have been implied in the earlier cryo-EM studies [1].

Using the existing X-ray crystallographic structures [1–3] and molecular modeling techniques we have built a full atomic structure of complexes formed by E. coli RecX and RecA proteins on a single-stranded oligonucleotide and performed molecular dynamics simulations on these complexes for 100 ns in a periodic water box. Formation of the RecX::RecA::ssDNA filament in solution was confirmed by SANS measurements. In the absence of RecA we observed formation of RecX::ssDNA filaments which geometry likely corresponded to a sandwich-like structure formed by pairs of RecX molecules on ssDNA. The SANS spectra computed from the molecular dynamics trajectory of the corresponding complexes using g_sans GROMACS module [4] were in a good agreement with the experiment.

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References

SW02.S7–67

Short peptides which enhance the fibrillogenesis of the model peptide

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Amyloid fibrils formation is the process that is common in human pathology. Recent studies show that some traditionally unrelated to amyloidosis diseases like hereditary form of glaucoma or influenza infection include the stage of amyloid-like oligomerization and amyloid fibrils formation. Unfortunately, the means against such protein oligomerization remain unknown.

The mechanism of amyloid-like fibrils and oligomers formation are very similar for different proteins. In previous work we show that peptide GYDTQAIVENNESTEGY (WT), corresponding 35–51 fragment of human alpha-lactalbumin (HAL) can form amyloid-like fibrils which can stimulate HAL oligomerization and fibrillogenesis in physiological conditions in vitro.

We use WT peptide solution as a model system for fibrillogenesis process study. N- and C- termini (GYDTand TEYG) of the peptide was the subject of our particular interest because of possible role of peptides primary structure symmetry in fibrillogenesis. We found that the fibrillogenesis of peptide WT can be dramatically enhanced by adding of tetrapeptide GYDT (L), identical to its N-terminus or tetrapeptide TDYG (R), which is homologous to its C-terminus. We investigated this process using the molecular dynamic simulation and found that peptides L and R can stabilize the beta-structure conformation of WT monomer. The mechanism of short peptide-mediated fibrillogenesis stimulation is hypothesized. Generation of products of spontaneous proteolysis products was shown in vitro by analysis of components of WT-peptide solution during the fibrillogenesis process by mass-spectrometry. Possible mechanisms and role of such processes in protein metabolism is discussed.

SW02.S7–68

Study of molecular and immunological features of novel plant lipid transfer proteins

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Plant lipid transfer proteins (LTPs) are small basic proteins that are capable to bind and transfer lipids in vitro. LTPs belong to a family of pathogenesis-related proteins and are considered to be involved in plant response to abiotic and biotic stresses, cutin and suberin formation, somatic embryogenesis, and signaling. Numerous plant LTPs display antifungal, antibacterial and antiviral activity and inhibit proteolytic enzymes. Different LTP isoforms are expressed in various organs and tissues. Besides, many LTPs are identified as food, pollen and latex allergens.

We discovered and isolated novel lipid transfer proteins in seeds of the lentil Lens culinaris (Lc-LTP1-8) and the pea Pisum sativum seeds (Ps-LTP) and in leaves and stalks of the garden dill Anethum graveolens (Ag-LTP). All the purified LTPs contain eight conservative cysteine residues forming four disulfide bonds. Molecular cloning and cDNA sequencing showed that the precursors of the isolated LTPs consisted of signal peptides and mature proteins. Recombinant analogs of the Lc-LTP1 and Ag-LTP were overexpressed in Escherichia coli as fusion proteins contained N-terminal histidine octamer and thioredoxin A. The purified recombinant LTPs were shown to inhibit growth of some phytopathogenic bacteria and fungi. As many other LTPs in plants, the lentil Lc-LTPs were observed to bind fatty acids. It was found that the lentil Lc-LTP1-3 are cross-reactive food allergens.

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SW02.S7–69

Identification and characterization of soybean endoplasmic reticulum oxidoreductin

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Endoplasmic reticulum (ER) is the intracellular compartment of the secretory pathway. Most of secretary and membrane proteins enter the ER and are subjected to disulfide bond formation. Protein disulfide isomerase (PDI) family proteins catalyze the disulfide formation in the ER. Two cysteine residues of the active center of PDI family proteins must be oxidized to form disulfide. In yeast and mammal, ER oxidoreductin 1 (Ero1) oxidizes PDI, which is a representative PDI family protein. We isolated soybean Ero1 (GmEro1) cDNA and expressed the recombinant GmEro1 (rGmEro1) in E. coli. The rGmEro1 was purified from E. coli lysate by affinity chromatography and gel filtration chromatography. The rGmEro1 was shown to be a FAD-binding protein by spectrophotometric analysis. Anti-GmEro1 antiserum was generated by immunization with the purified rGmEro1. Western blot analysis with the antiserum revealed that GmEro1 was expressed in root, stem, leaf and cotyledon. Maximum expression of GmEro1 and GmEro1 mRNA in the cotyledon were observed in the period when the synthesis of the seed storage proteins was the highest. GmEro1 was an N-glycosylated membrane bound protein. We found that rGmEro1 consumed O₂ in the presence of glutathione and recombinant soybean PDI family proteins such as GmPDIL-1, GmPDILM, GmPDIS-I or GmPDIS-2, suggesting that GmEro1 is a flavoprotein oxidase and catalyzes the oxidation of the active centers of these PDI family proteins. In addition, GmPDIL-1, GmPDILM, GmPDIS-I and GmPDIS-2 catalyze the refolding of the denatured and reduced RNase A to the nature structure in the presence of rGmEro1 without any redox buffer.

SW02.S7–70

Structure of uridine phosphorylase from Shewanella oneidensis MR-1 in the free state at atomic resolution and its structure in complex with the natural substrate

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Uridine phosphorylase (UDP, EC 2.4.2.3), a key enzyme in the pyrimidine salvage pathway, catalyzes the reversible phosphoryolysis of uridine (Urd) to uracil and ribose 1-phosphate. The gene expression of UDP from Shewanella oneidensis MR-1 (SoUDP), which is a free-living gram-negative γ-proteobacterium and is a facultative anaerobe capable of surviving and proliferating in both aerobic and anaerobe capable of surviving and proliferating in both aerobic and anaerobic conditions.
Human superoxide dismutase 1 (hSOD1) maturation through interaction with human copper chaperone for SOD1 (hCCS)

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Introduction: Superoxide dismutase 1 (SOD1) is a copper enzyme that catalyzes the dismutation of the toxic superoxide anion, a byproduct of cellular respiration, to molecular oxygen and hydrogen peroxide. Although mature eukaryotic SOD1 is a remarkably stable homodimer, the nascent monomer requires several posttranslational modifications before reaching the active dimeric form. Copper chaperone for SOD1 (CCS), is the physiological partner for the complex mechanism of SOD1 maturation. SOD1 metalation in human cells is carried out with the assistance of CCS, which probably also exhibits disulfide isomerase activity. However, the molecular mechanism of CCS-dependent maturation of SOD1 is still unknown. The goal of this work is to elucidate the CCS-dependent mechanism of SOD1 maturation at the molecular level.

Results: We used the synergy between electrospray ionization mass spectrometry (ESI-MS) and NMR to study this process in vitro. We report a model for human CCS-dependent SOD1 maturation based on the study of the interactions of human SOD1 (hSOD1) with full-length WT human CCS (hCCS), as well as with hCCS mutants and various truncated constructs comprising of one or two of the protein’s three domains. We showed that hSOD1 reaches its mature copper-loaded form in vitro only when the disulfide-reduced zinc-bound form of hSOD1 is treated with Cu(I)-bound full-length hCCS, whereas truncated forms of hCCS are not able to produce a mature dimeric Cu,Zn-hSOD1 protein.

Conclusion: Domain 1 of hCCS is necessary to load hSOD1 with Cu(I), requiring the heterodimeric complex formation with hSOD1 fostered by the interaction with domain 2. Domain 3 is responsible for the catalytic formation of the hSOD1 Cys-57 – Cys-146 disulfide bond, which involves both hCCS Cys-244 and Cys-246 via disulfide transfer.
SW02.S7–73
Key role for membrane lipids in orchestrating the endocannabinoid hydrolase (FAAH) function and subcellular localization
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The influence of lipid composition on the enzymatic activity is important for membrane-bound proteins, yet its basic principles remain largely unknown. Recently, we reported a small angle X-ray scattering (SAXS) study demonstrating that the structure of FAAH, a prototypical example of membrane enzymes terminating the signalling of the endocannabinoids, is organized as an octamer of dimers, and, in the presence of detergents mimicking the membrane milieu, it dissociates to dimers [1]. Here, we further extend this study, by using different preparations of isolated cell membranes, demonstrating that membrane lipids are important in orchestrating the subcellular localization and activity of FAAH. We report that FAAH has a preferential interaction and higher enzymatic activity within membranes containing both cholesterol and the natural FAAH substrate, anandamide (AEA). Additionally, confocal microscopy shows a colocalization of cholesterol and AEA, as well as of AEA and FAAH, in mouse neuroblastoma cells. Membrane binding experiments by fluorescence resonance energy transfer (FRET) and computational analysis suggest a mechanism through which cholesterol increases the accessibility of FAAH active site to the substrate.

The role of cholesterol in modulating the function of FAAH constitutes a conceptual step forward to underpin the functional homodimers in this class of enzymes are not tightly packed as in the crystal structure, but they have a flexible conformation that could account for the surface flexibility required by these enzymes to allow the substrate access to the active site.

SW02.S7–75
Monitoring temperature-induced local conformational changes in mammalian tyrosyl-tRNA synthetase by fluorescent probes
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Tyrosyl-tRNA synthetase (TyrRS) is one of the key enzymes of protein biosynthesis, which catalyzes the highly specific aminocytlation of homologous tRNA(Tyr). Eukaryotic TyrRS as a multifunctional protein is involved in some noncanonical functions, including associated with neurodegenerative diseases such as neuropathy Charcot-Marie-Tooth type 2. It is known that in the mutant proteins in neurodegenerative diseases, protein folding can stop at the stage of molten globule. The molten globule state is characterized by higher exposure of hydrophobic regions, which allows to study this state by fluorescent probes.

The aim of this work is to study the local conformational changes in N-terminal catalytic module TyrRS (mini-TyrRS) and their ability to transition into molten globule state.

Methods: Recombinant proteins were obtained by bacterial expression in E. coli BL21(DE3)pLysE by standard methods. Conformational mobility of mini-TyrRS in the temperature range 20–60°C was studied by monitoring of parameters of protein tyrotophan fluorescence. A fluorescent probes 1,8-ANS and bis-ANS has been used. In order to find the binding regions of probes at the surface of HS TyrRS, the molecular docking of 1,8-ANS and bis-ANS to the enzyme surface was performed in silico. Computational modeling was performed with AutoDock Vina program.

Results: With increasing temperature to 42–45°C, the changes of tyrophophan fluorescence maximum were up to 344 nm for mini-TyrRS which are associated with local conformational changes in protein and possible formation of molten globule. The interaction of 1,8-ANS and bis-ANS with mini-TyrRS resulted an increase of fluorescence intensity and shifts of the maximum emission to the short wave region, due to the binding of probe to the hydrophobic sites on the protein surface. Computer simulation of molecular docking of 1,8-ANS and bis-ANS to the HS/TyrRS revealed several binding sites, one of which is common for the both dyes. The common region was formed by Arg16, Lys265, Phe269, Thr290, Tyr292, and the Ser273, Ala291, Val293 for bis-ANS only. The energy of interactions of 1,8-ANS and bis-ANS to this hydrophobic region of TyrRS was about −6.1 and −5.7 kcal/mol, respectively.

Conclusion: Fluorescence spectroscopy and molecular docking allow to detect the formation of hydrophobic sites on the surface in N-terminal catalytic module TyrRS.
SW02.S7–76
Unique alpha-helical insert between the N-terminal domain and AAA* module of LonA proteases
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Homooligomeric LonA proteases are the key components of the protein quality control system in bacteria and eukaryotes. Structural data for the individual domains and/or their combinations in several LonA representatives have recently become available. A comparative analysis of the primary, secondary, and tertiary structures of various LonA proteases revealed the presence of a long alpha-helical insert between the N-terminal domain and the AAA* module. This insert appears to be common to all known members of the LonA subfamily. Separately obtained structural data for the two fragments comprising this insert lead to a hypothesis that this region is reminiscent of the alpha-helical domain of the AAA*-1 module of the chaperone-disagregase ClpB/Hsp104, including a coiled-coil insert. Thus, LonA proteases presumably represent a unique subfamily of AAA* proteins, which, in addition to their single AAA* module, also contain a part of a second AAA* module which is present in full length in the proteins that belong to the ClpABC family from the HslU/ClpX/Lon/ClpABC clade.

SW02.S7–77
The X-ray study of the trigonal crystal form of phosphopantetheine adenylyltransferase from Mycobacterium tuberculosis
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Phosphopantetheine adenylyltransferase from Mycobacterium tuberculosis (PPAT Mt) is involved in the coenzyme A (CoA) biosynthesis, catalyzing the penultimate step of the process, resulting in the formation of the dephosphocoenzyme A (dPCoA) from 4′phosphopantetheine (PhP) and ATP. Reduction of the intracellular level of CoA prevents the bacterium growth. Therefore PPAT that catalyses the key step of CoA biosynthesis is a suitable therapeutic target for the rational drug design.

To date the 3D-structures of apo PPAT and PPAT complexed with some of functional ligands (ATP, CoA, dPCoA, PhP) have been solved. The structural mechanism of PPAT Mt action was studied recently by X-ray using crystals of PPAT Mt complexed with CoA, dPCoA and ATP [1]. Different kinds of crystals contained one, two or four molecules in the asymmetric unit.

In the presented study a new, trigonal crystal modification of PPAT Mt was grown in microgravity in capillaries by counter-diffusion method in the presence of low concentrations of ATP. Grown crystals belonged to P32 space group and contain hexameric PPAT molecule in the asymmetric unit. The 3D-structure of the trigonal crystals was solved at 1.85 Å resolution based on the X-ray diffraction data collected at SPring-8 synchrotron facility. The crystal packing and its influence on the conformation of the enzyme molecule have been studied through the comparison of different crystal forms.

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Reference

SW02.S7–78
Studies of the interaction of yeast and human HMGB with AT-rich DNA
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HMG proteins act as architectural proteins that affect cellular functions by modulating chromatin structure and thereby gene expression in eukaryotic cells. HMG proteins are one of the three members of this family and are defined by their DNA binding motifs: the HMG-boxes. The HMG-box domain, found also in other proteins, has an L-shaped fold consisting of three alpha-helices.

HMG proteins contain one or more HMG-boxes and have little or none sequence specificity for DNA binding. However, recent studies have shown that yeast HMGs can present specificity for some AT-rich sequences (Zhu et al, Genome Res. 2009 19: 556–566). To further address this question, we have used electrophoresis mobility shift assays (EMSA) using one HMG protein, human HMGB1 and yeast NHP6A and different oligonucleotides (mostly AT-rich oligonucleotides). In the case of HMGB, we have worked with fragments corresponding to the two HMG-box domains (Box-A and Box-B) and also with a fragment containing both domains. The assays show some preferential binding. We have also confirmed a different contribution of both domains to the interaction.

Furthermore, we have performed crystallographic assays with these three fragments of the HMGB1 protein and NHP6A with several of the oligonucleotides. We have obtained crystals of diverse morphology and we show the preliminary diffraction data obtained.

SW02.S7–79
Detailed kinetic analysis of interaction between the FOXO4-DNA-binding domain and the DNA
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The FOXO forkhead transcription factors are potent transcriptional activators involved in a wide range of key biological processes. FOXO proteins recognize their consensus sequences through direct and water-mediated side chain-base contacts while numerous nonspecific contacts to the DNA backbone seem to be important for the overall stability of FOXO-DNA complexes. The binding of FOXO proteins to the target DNA is regulated by means of post-translational modification or protein–protein interactions and fine variations in FOXO’s DNA-binding affinity are an important factor involved in the regulation of target gene expression. In this work, the significances of individual polar contacts between FOXO4-DNA-binding domain (FOXO4-DBD) and the double
stranded DNA containing the insulin-responsive (IRE) and the Daf-16 family member-binding (DBE) elements were determined by using surface plasmon resonance measurements. The results revealed that the interaction between FOXO4-DBD and DNA is preferably described by using a two-state binding model which indicates a conformational change of FOXO4-DBD upon binding to DNA. Alanine scanning of amino acid residues engaged in contacts between FOXO4-DBD and the DNA showed that none of these residues is absolutely critical for the overall stability of the FOXO4-DBD-DNA complex, but they rather form a complex network of direct and water-mediated side-chain-base contacts with additive effect on complex stability. Our data also show that all parts of the DNA-binding surface of FOXO4-DBD are important for the stability of the complex and that certain nonspecific contacts with the DNA backbone are very important for the binding affinity as well as for the binding specificity of FOXO4 transcription factor.

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References

**SW02.S7–80**

The 14-3-3 protein binding-dependent structural modulation of phosducin

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Phosducin (Pdc), a highly conserved phosphoprotein, plays an important role in the regulation of G protein signaling, transcriptional control, and modulation of blood pressure. Pdc is negatively regulated by phosphorylation followed by binding to the 14-3-3 protein, whose role is still unclear. To gain insight into the role of 14-3-3 in the regulation of Pdc function, we studied structural changes of Pdc induced by phosphorylation and 14-3-3 protein binding using time-resolved fluorescence spectroscopy. Our data show that the phosphorylation of the N-terminal domain of Pdc at Ser-54 and Ser-73 affects the structure of the whole Pdc molecule. Complex formation with 14-3-3 reduces the flexibility of both the N- and C-terminal domains of phosphorylated Pdc, as determined by time-resolved tryptophan and dansyl fluorescence. Therefore, our data suggest that phosphorylated Pdc undergoes a conformational change when binding to 14-3-3. These changes involve the G(beta gamma) binding surface within the N-terminal domain of Pdc, and thus could explain the inhibitory effect of 14-3-3 on Pdc function.

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References
Assembly of Schistosoma mansoni septins into hetero-oligomeric complex and biophysical characterization of its subunits

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Schistosoma mansoni is one of the main causative agents of schistosomiasis, a neglected tropical disease affecting over 230 million people in developing and underdeveloped countries. The recent publication of its genome has enabled the identification of proteins and new potential drug targets.

Septins are a family of GTPase proteins involved in a variety of cellular functions such as cytokinesis, vesicle trafficking, diffusion barriers, and scaffold. However, the mechanism by which septins participate in these processes is still poorly understood. The number of septin members is variable in different organisms, but the ability to self-assemble into hetero-oligomeric complexes that further polymerize into filaments is unanimous.

We have identified four genes encoding septins in the S. mansoni genome. Multiple sequence alignment and phylogenetic analysis of S. mansoni and human septins were performed. The human septins are classified into four subgroups; interestingly the S. mansoni septins possess representatives in the three subgroups that comprise the best well known human septin complex (SEPT2–SEPT6–SEPT7). The basal position of S. mansoni septins in relation to more ramified branches of human septins suggests that S. mansoni display a simpler configuration, probably similar to an ancestral configuration. Curiously, the only gene duplication within a septin group observed in S. mansoni is from SEPT7 group, which is not paralleled in humans; that contain only one protein in this group. In order to verify the S. mansoni septins assembly, a co-expression system was designed. SmSEPT7.2 was fused with a His-tag and SmSEPT5 and SmSEPT10 were co-purified heterologously, indicating that these proteins indeed interact. The presence of the three proteins into the complex was assessed by SDS-PAGE and mass spectrometry. As far as we know, this is the first report of a septin complex in the Platyhelminthes phylum. Additionally, two S. mansoni septins (SmSEPT5 and SmSEPT10) were expressed separately and then subjected to a set of biophysical approaches, revealing their structural stability, GTPase activity, and nucleotide binding properties.

Novel affinity medium for purification of the human beta-adrenergic receptors

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G-protein coupled receptors (GPCRs) are a large family of about 800 transmembrane proteins. All GPCRs molecules consist of polypeptide chain with seven transmembrane helix segments. GPCRs are taking part in signal transduction through the cell membrane and regulating numerous significant physiological processes. For structural and functional investigations, relatively large amounts of functionally active receptor are needed. Expression of GPCRs in E. coli provides a method which yields milligram quantities of these receptors in inactive inclusion bodies. It means that these proteins have to be solubilized, folded and then purified. The most effective way to perform activity tests and following purification of the correctly folded receptor is affinity chromatography on the ligand-activated sorbents. Here we provide a new method of preparation chromatography matrix with covalently attached receptor’s antagonist alprenolol for purification of beta-adrenergic receptors.

For this, alprenolol-cysteamine containing the primary amino group was synthesized by radical addition of cysteamine to the double bond of alprenolol. Alprenolol-cysteamine was coupled to the commercially available epoxy- and carbosyl-activated agarose gels in one step procedure. Importantly, that amount of alprenolol bound with matrix can be controlled by UV spectroscopy. To test the properties of the obtained matrices with attached ligand we tested incorporated mutant of beta-2-adrenergic receptor (beta2AR), in which cysteines from the transmembrane domains were replaced by the structural similar residues valine and serine, was incorporated into phospholipids nanodiscs. As a control, we used nanodiscs with incorporated structural analog of GPCRs – bacteriorhodopsin from Exiguobacterium sibiricum (ESR). Nanodiscs with ESR didn’t bind to the affinity-column when concentration of alprenolol bound with the matrix was lower than 1 mM. In the same time, nanodiscs with beta2AR selectively bound to the chromatographic medium with immobilized alprenolol and wash through from the column only with buffer contained free alprenolol.

Thus, a new, simple and effective method for affinity purification of beta-adrenergic receptors was developed.

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Lysostaphin and LytM – how similar and how different are these two peptidoglycan hydrolases

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Staphylococcus aureus is an important pathogen which can cause a range of illnesses from minor skin infections to life-threatening diseases. Difficulties in development of potent treatment for these infections and effective elimination of the bacterial colonization are related not only to the robust architecture of Gram positive cell walls and biofilm formation but also to spreading drug resistance.

Enzybiotics are an attractive alternative to antibiotics and as such attract more and more attention as a powerful but still unexplored weapon against bacterial infections. The term ‘enzybiotic’ is used to describe any enzyme that displays general antibacterial or antifungal properties, including exolysin and autolysins. Lysostaphin is the best studied exolysin (bacteriocin) currently tested in various medical applications. It is a glycin-glycine hydrolase secreted by S. simulans that cleaves the S. aureus peptidoglycans leading to cell lysis. LytM is an autolysin produced by S. aureus which has the same specificity as lysostaphin and can also lead to cell lysis when applied from outside. These two enzymes have a modular structure, are produced as preprenzymes and need processing for activation. The only domain they share with around 50% identity is a 130 aa long catalytic domain. Additionally, lysostaphin has a cell wall targeting domain (CWT) which is absent in LytM.

Although these two enzymes have the same specificity, they differ greatly in their response to various reaction conditions. Lysostaphin and LytM activities depend on ionic strength in a very different way. Lysostaphin degrades cell walls inefficiently in low conductivity buffers. In contrast, LytM works best at low conductivity, and is almost ineffective in high conductivity buffers. Moreover, LytM shows unusually high efficacy in the wide range of temperatures; even in just above 0°C it sustains over 60% of optimal activity. In contrast, lysostaphin activity is limited to a much narrower range of temperatures.
In our comparative biochemical and structural studies of LytM and lysostaphin we try to explain the nature of these differences and provide a background for speculations about the mechanisms of regulation of enzyme activity by environmental factors such as ionic milieu and temperature.

**SW02.S7–85**

Heat-induced structural dynamics of a thermoacidophilic small heat-shock protein sHSP14.3; functional implications

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The small heat shock proteins (sHSPs), are ATP independent molecular chaperones, that bind to and stabilize stress denatured proteins in order to protect them from aggregation and promote their refolding. The members of this family are characterized by sharing a conserved motif so called α-crystallin domain (ACD) in the C-terminal region which displays sequence similarity to the primary protein component of the vertebrate eye lens. The monomeric molecular masses of sHSPs range from 12 to 43 kDa. Interactions between two monomers at ACDs define a dimeric building block and itself aggregates into large oligomeric assemblies. Available crystallographic, cryoEM and spin labeling EPR analyses revealed highly dynamic oligomers of different sizes and compositions. The oligomeric heterogeneity is further enhanced by substrate binding and increased temperature, which complicate the structural investigations. In this study we investigated temperature controlled chaperone function of a sHSP, Tpv-HSP 14.3 from thermoacidophilic archeon *Thermoplasma volcanium* GSS1 using bovine l-glutamic dehydrogenase (boGDH) (a mesophilic enzyme with an optimum temperature of 25°C and inactivated rapidly at 56°C) as the model substrate. Thermal unfolding/recovery assays revealed that Tpv-HSP 14.3 maintained boGDH in enzymatically active state under heat-stress, i.e., 53°C. Although boGDH activity was drastically reduced (up to 8.3%) after heat-inactivation at 53°C, the residual activity was increased approx. two-fold when the enzyme samples were treated with heat in the presence of Tpv-HSP 14.3. We applied dynamic light scattering technique to investigate structural changes in Tpv–HSP 14.3 during its chaperone action as a function of temperature. We showed that Tpv-HSP 14.3 exists as a heterogeneous oligomeric ensemble at room temperature. But, the oligomers underwent extensive structural changes and became much larger at higher temperatures. The oligomers size increased even more when the sHSP complexed with target proteins. Our results were consistent with earlier made predictions from studies of other sHSPs including α-crystallin.

**SW02.S7–86**

Statistical potential for identification of 2+ metal cations bound in proteins

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Metal ions are play different and important functions in protein. For example, with according to ‘ionic hypothesis’ breaking of ion binding site may lead to different disease – Parkinson disease, Alzheimer disease etc. Desing protein which bind defined ion may be very interesting for obtaining of rare-earth metals. By this reason task of prediction type and position binding site of ions are very important. Early, this task solved by finding of useful template from database of known structures, which are selected by means of 3d or classical alignment. We developed algorithm for detection ions in user’s protein structures, based on empirical potential method.

Empirical potential is a statistical construction, which are presented ratio of observed and expected frequencies of contact between atoms of structure and current ion. Observed frequency was obtain from database of known structures, expected frequency is estimate from method Monte Carlo Reference State (MCRS). With using of PDB databases we build are empirical potential for most popular ion types (calcium, zinc, magnesium etc). With help this potential can compute pseudoenergy of binding in ever point near protein. Point with maximum energy of binding is most probably position in ion. If in protein structure conduct prediction for different type of ions we have to choose type of ion, which pseudoenergy of binding are more than another.

We test our software on set from 99 structures, which are not was included in learning set. (33 structure for zinc, 33 for calcium, 33 for magnesium).

Our method shows are good accuracy in best case (for calcium and zinc) equal approximately 90% of correct prediction type of ion with level of false positive prediction equal approximately 15%.

**SW02.S7–87**

Dissociation of the subunits of the calcium-independent receptor of α-latrotoxin (CIRL1)

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CIRL1 also called latrophilin 1 or CL1 belongs to the family of adhesion G protein-coupled receptors (GPCRs). As all members of adhesion GPCR family CIRL1 consists of two heterologous subunits, extracellular hydrophilic p120 and heptahelical membrane protein p85. Both subunits are encoded by the same gene and represent products of intracellular proteolytic processing of the CIRL precursor in the GPS domain (GPCR Proteolytic Site). The mature receptor is a noncovalently associated two-subunit complex expressed on the cell surface. It has been recently proposed that two fragments of CIRL p120 and p85 remain membrane bound but dissociate and behave as independent cell surface proteins.

We tested the possibility that the two-subunit complex of adhesion GPCR can dissociate under physiological conditions. We showed that a minor portion of the CIRL1 receptor complexes dissociates in vivo, producing the soluble receptor ectodomain, and this dissociation is due to the further cleavage of CIRL1 by a second protease. We found that the site of the second cleavage is located in the short N-terminal extracellular tail of p85, between the GPS domain and the first transmembrane segment of CIRL1. Our data suggest that the two-step proteolytic processing may represent a regulatory mechanism that controls cell surface expression of membrane-bound and soluble forms of CIRL. Using chimeric constructions of CIRL1 we evaluated the amount of p120–p85 complex still presented on the cellular membrane and confirmed that on the cell surface major amount of mature CIRL1 presented as a p120–p85 subunit complex.

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**SW02.S7–88**

Fluorone dyes binding to extracellular and cytoplasmic domains of Na,K-ATPase

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Combination of fluorescence techniques and molecular docking was used to monitor interaction of Na,K-ATPase and its large cytoplasmic loop connecting fourth and fifth transmembrane helices (C45) with fluorone dyes (i.e. eosin Y, 5(6)-carboxy eosin, rose bengal, fluorescein, and erythrosine B). Our data suggested that there are at least two binding sites for all used fluorone dyes, except of 5(6)-carboxy eosin. The first binding site is located on C45 loop, and it is sensitive to the presence of nucleotide. The other site is located on the extracellular part of the enzyme, and it is sensitive to the presence of Na+ or K+ ions. The molecular docking revealed that in the open conformation of C45 loop (which is obtained in the presence of ATP) all used fluorone dyes occupy position directly inside the ATP-binding pocket, while in the closed conformation (i.e. in the absence of any ligand) they are located only near the ATP-binding site depending on their different sizes. On the extracellular part of the protein, the molecular docking predicts two possible binding sites with similar binding energy near Asp897(α) or Gln69(β). The former was identified as a part of interaction site between α- and β-subunits, the latter is in contact with conserved FXYD sequence of the γ-subunit. Our findings provide structural explanation for numerous older studies, which were performed with fluorone dyes before the high-resolution structures were known. Further, fluorone dyes seem to be good probes for monitoring of intersubunit interactions influenced by Na+ and K+ binding.

**SW02.S7–89**

The effect of substitutions of E457 and A534 residues in thermostable mutant of Luciola mingrelica firefly luciferase on its activity and stability

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Firefly luciferase catalyzes firefly luciferin oxidation by O2 in presence of ATP. It is widely applied in various bioanalytical systems and in biochemical and physiological studies. The enzyme is composed of a large N-domain (residues 1–445) and a small C-domain (residues 446–548). The roles of amino acid residues of N-domain are extensively investigated. Meanwhile information on the roles of C-domain amino acid residues is very limited.

E457 residue is highly conserved in the enzymes of ANL superfamily, including firefly luciferase. To clarify its role in firefly luciferase L. mingrelica we obtained mutants with following single mutations on the basis of its thermostable form 4TS: E457V, E457Q, E457D, E457K. In the molecules of pH-independent luciferases E457 residue forms two additional hydrogen bonds with R534, which are absent in 4TS and its above-mentioned mutants. To investigate the role of these bonds two mutants were obtained: in A534R mutant the H-bonds can be formed, while double E457V/A534R mutant demonstrates the effect of A534R, which is not related to the H-bond formation.

All the mutants were purified to homogeneity and extensively characterized. All mutants except E457D preserved a high level of enzymatic activity. Km(ATP) values of E457K, E457D, E457V and A534R were 2–3-folds higher than that of 4TS, Km (LH2) values were 2–3-folds lower for all the mutants except E457K. The change of sign (E457K) or total elimination (E457V) of the charge on the 457th residue lowers the enzyme thermostability by the factor of ~2. The substitution, preserving the charge on the residue (E457D), results in a smaller destabilizing effect. Introduction of additional hydrogen bonds between E457 and R534 residues (A534R) results in two-fold increase of thermostability in comparison with that of 4TS.

The contribution of low-energy red emitter in an overall bioluminescent spectrum grows with the increase of the temperature for most of the mutants, but its role is greater for the less thermostable enzymes. Meanwhile high-energy green emitter plays more significant part in the spectral characteristics of 4TS and its stable mutants (A534R and E457D). Possible explanations of the above-described effects in relation to the structural changes in luciferase molecule are proposed.

**SW02.S7–90**

A novel tool to shield against α-synuclein’s toxic effects

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The unstructured and aggregation-prone protein α-synuclein is linked to the pathology of a group of neurodegenerative disorders, for example Parkinson’s disease, which are marked by the presence of Lewy bodies. The oligomeric and fibrillar forms of the protein are implicated as the main culprits in the process.

Here in this work the engineered binding protein Zsyn69, isolated from a combinatorial protein library via the phage display system, is employed to study its potential in blocking or mitigating the toxic effects of α-synuclein’s fibrils and oligomers in a cell culture model. The binding protein is designed to remain functional in the cell culture milieu. Interaction of the binding protein towards α-synuclein was initially confirmed by ITC (Isothermal Titration Calorimetry), showing desirable binding affinity. α-synuclein’s aggregation kinetics was analyzed by Thioflavin-T fluorescence, demonstrating thorough inhibition of aggregation at equimolar concentrations of Zsyn69. The MTT cell viability test was used to characterize the ability of Zsyn69 in controlling the toxicity of α-synuclein fibrils in SH-SY5Y neuroblastoma cell line. Our results revealed significant reduction of toxic effect of the aggregates in the presence of the binding protein.

**SW02.S7–91**

Amyloid beta 1-42 oligomerization in vitro and characterization with SDS-PAGE, MALDI and ESI MS

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Aggregation of amyloid beta (Ab) peptides into oligomers and amyloid plaques in human brain is considered to be a causative factor in Alzheimer’s disease (AD), however, the precise mecha-
nism behind the AD onset and the contribution of Ab oligomers to AD pathology has remained elusive.

The current investigation was focused on the preparation of Ab oligomers in vitro and their comparative characterization with SDS-PAGE, gel filtration, matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) and electrospray ionization mass spectrometry (ESI MS).

Interesting phenomenon was that Ab42 seems to form oligomers when the peptide is dissolved in organic solvents like acetone, ethanol, methanol and 2-propanol. We studied the stability and kinetics of formation of those oligomers using SDS-PAGE. We observed that incubation of Ab42 in the presence of SDS (2 and 4 mM) results in increased level of tetramer compared with other SDS concentrations. Interestingly, the presence of oligomer bands depends on the heating of the probe, which results in the disintegration of oligomers into monomers after 30 s and reintegration after 20 min. We solubilized oligomers from the SDS-PAGE bands and analyzed obtained samples with MALDI MS, which detected only Ab monomers from these samples indicating that oligomers are non-covalently bound.

According to the literature, oligomerization can be induced in vitro also by using incubation of Ab42 in the presence of SDS and subsequent SDS removal by dialysis. We prepared and characterized also those SDS-induced Ab oligomers with MALDI and ESI MS. Unfortunately we detected only monomers, which confirms previous hypothesis about non-covalent nature of formed Ab oligomers.

Results showing that the Ab oligomers are observed in SDS-PAGE and gel filtration (in the presence of SDS) but not in MALDI and ESI MS implicate that the low molecular weight Ab oligomers are not covalently bound and are induced by organic solvent or SDS.

**SW02.S7–92**
The molten globule state is the single conformational state of high lability

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The intermediate molten globule state is observed when studying protein folding. Transition from the native protein structure to the molten globule state may be caused by various factors, including a decrease of pH, proximity to phospholipid membranes, and moderate concentrations of denaturants. For sperm whale apomyoglobin, it is shown that this state is accumulated at pH 4.0–4.2 and partially at urea concentration around 3M at pH 6.2. Besides, after heat denaturation the native protein conformation undergoes transition into an intermediate state with molten globule-like properties. To understand whether intermediate states are independent from one another, we constructed a diagram of conformational states of apomyoglobin. The diagram covers a wide range of conditions where these states are accumulated. It is shown that the native, intermediate, and unfolded states of apomyoglobin have different far UV CD spectra and those of tryptophan fluorescence. These properties were used to observe accumulation of this or that state under certain conditions. As a result, a 3D diagram of conformational states of apomyoglobin was constructed in coordinates of pH-temperature-urea concentration. An analysis of the resultant diagram has shown the following: first, conditions favorable for accumulation of the intermediate states are integrated into a common area of the diagram; second, we observed no dramatic changes of spectral properties under changed conditions in this area. This allows a conclusion that the area of this conformational state is continu-

ous, and the state itself is thermodynamically uniform over the entire range of its accumulation.

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**SW02.S7–93**
Right- and left-handed three-helix proteins: experimental and simulation analysis of differences in folding and structure

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Despite the large number of publications on three-helix protein folding there is no study devoted to the influence of handedness on the rate of three-helix protein folding. From the experimental studies we make a conclusion that the left-handed three-helix proteins fold faster than the right-handed ones. What may explain this difference? An important question arising in this paper is whether the modeling of protein folding can catch the difference between the protein folding rates of proteins with similar structures but with different folding mechanisms. To answer this question, the folding of six three-helix proteins (four right-handed and two left-handed), which are similar in size, was modeled using the Monte Carlo and dynamic programming methods. The studies allowed us to determine the orders of folding of the secondary-structure elements in these domains and amino acid residues which are important for the folding. The obtained data are in good correlation with each other and with the experimental data. Structural analysis of these proteins demonstrated that the left-handed domains have a lesser number of contacts per residue and a smaller radius of cross section than the right-handed domains. This may be one of the explanations of the observed fact. The same tendency is observed for the large dataset consisting of 332 three-helix proteins (238 right- and 94 left-handed). From our analysis, we found that the left-handed three-helix proteins have some less-dense packing that should result in faster folding for some proteins as compared to the case of right-handed proteins.

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**SW02.S7–94**
Effect of resveratrol and tiron radicals on the activity of glyceraldehyde-3-phosphate dehydrogenase

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Oxidative stress may occur in cells in the event of an imbalance between the production of reactive oxygen and nitrogen species and the enzymatic and non-enzymatic processes leading to their elimination. Among oxygen radicals, superoxide anion radical is considered to be very important, because it is the first oxygen radical formed by the capture of an electron by the oxygen molecule. It can be transformed into other oxygen radicals or peroxynitrite by successive reactions.

Oxidative stress first of all results in damage to proteins due to their high cellular content and their high reactivity with reactive species. Protein oxidation and aggregation may lead to a number of diseases, including neurodegenerative ones (e.g. Parkinson’s and Alzheimer’s). The protein that is known to be one
of the major targets of oxidative stress is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is found to play a vital role in several neurodegenerative disorders. Some authors suggest that the preservation of GAPDH structure and activity may be a promising therapeutic target in slowing or halting neurodegeneration in aging-associated.

Literature data show that both resveratrol and tiron effectively scavenge superoxide anion radical. The reaction of RSV with the superoxide anion radical leads to the formation of the alkoxyl radical of resveratrol, preferentially located at position 4′ in the phenol ring. Similarly, superoxide anion scavenging by tiron leads to the formation of the semiquinone radical.

In our previous work, we showed that the superoxide anion radical generated in the xanthine/xanthine oxidase system inactivates GAPDH to a considerable extent. This study shows that the increase inactivation of GAPDH in the xanthine/xanthine oxidase system in the presence of RSV or tiron was caused by secondary resveratrol or tiron radicals. The removal of superoxide radical by superoxide dismutase completely prevented inactivation both in the presence and absence of resveratrol or tiron. We have found that secondary tiron and resveratrol radicals are 3.5- and two-fold more effective in GAPDH inactivation than superoxide anion radical alone. In our experimental system, both resveratrol and tiron exhibit prooxidant properties.

**SW02.S7–95**

**Selection of an engineered binding protein to tau**

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The tau protein is involved in many neurodegenerative diseases, such as Alzheimer’s disease and frontotemporal dementia (FTD). In the brains of patients aggregates of tau, paired helical filaments, can be found post mortem. The core of tau fibrils is formed by four imperfect repeat domains at the C-terminus of tau. In order to gain insight into the structural biology of protein-protein interactions involving the natively unfolded protein tau, and to investigate the aggregation mechanism, we selected an engineered binding protein for tau by phage display. The combinatorial library was generated on the basis of a preselected an engineered binding protein for tau by phage display. As a target for this selection a construct comprising the protein tau, and to investigate the aggregation mechanism, we

**SW02.S7–96**

**Heterologous expression of Bacillus licheniformis VK21 lantibiotic system components in E. coli**


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Lantibiotics are small antimicrobial peptides produced by Gram-positive bacteria and characterized by specific posttranslational modifications: dehydrated serine and threonine residues and thioether bonds between the dehydrated residues and cysteins. In previous study, we discovered a novel two-component lantibiotic lichecidin, produced by Bacillus licheniformis, strain VK21. Its components, the peptides Lchz and Lcb, act synergistically displaying antibiotic activity against Gram-positive bacteria in the nanomolar concentration range.

The gene encoding enzyme LchM2, which catalyzes dehydration and cyclization of the prepeptide LchA2, was amplified and sequenced. The expression plasmids for heterologous production of the prepeptides LchA1 and LchA2 in Escherichia coli, along with the corresponding modifying enzymes, were constructed. The recombinant prepeptides were expressed as fusion proteins containing N-terminal histidine tags and protease factor Xa cleavage sites for further isolation of the mature peptides. The prepeptides LchA1 and LchA2 were purified from soluble and insoluble protein fractions by immobilized metal-chelate affinity chromatography (IMAC) and subsequent reversed-phase HPLC. The recombinant N-terminally His-tagged enzyme LchM1 was also expressed in E. coli and purified from the soluble protein fraction by IMAC. All the recombinant proteins were characterized by SDS-PAGE and MALDI-TOF mass spectrometry. The structure of the recombinant LchM1 was confirmed by tryptic digestion with subsequent MALDI-TOF mass spectrometry.

**SW02.S7–99**

**Dimerization of transmembrane domain of amyloid precursor protein in micellar environment**


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For appropriate therapy of Alzheimer’s disease which affects people all over the world regardless of nation and social status it is essential to determine the molecular basis of the pathogenesis. Amyloid beta-peptide which forms amyloid plaques in brain during Alzheimer’s disease is the product of sequential cleavage of a single-span membrane amyloid precursor protein (APP). More than half of mutations of APP found to be associated with familial forms of Alzheimer’s disease are located in its transmembrane domain. The pathogenic mutations presumably affect structural-dynamic properties of the APP transmembrane domain, changing its conformational stability and/or lateral dimerization. In the present study the structure and dynamics of recombinant peptide, corresponding to APP fragment Asp751-Lys726 including the APP transmembrane domain with adjacent N-terminal juxtamembrane sequence and metal binding site, were determined in membrane-emulating milieu composed of detergent micelles using NMR spectroscopy methods. The structure obtained in dodecylphosphocholine micelles consists of two alpha-helices: a short surface-associated juxtamembrane one Lys687-Asp694 and a long transmembrane one Lys699-Lys724, the helices are connected via a mobile loop region. A minor bending of the transmembrane alpha-helix takes place near tandem residues Gly708-Gly709. Binding of Zn2+ ions with metal-chelating region does not perturb the transmembrane dimerisation motif. The APP transmembrane alpha-helix self-associates in a left-handed parallel dimer through extended heptad repeat motif I176xxM178xxG180xxA182xxL184xxI186xxT188xxI190xxT192 whereas alternate tetrad motif G176xxG178xxG180xxG182 apparently assigned for interaction with cholesterol. The cholesterol-binding hydrophobic cavity is formed under the loop region where the juxtamembrane alpha-helix contacts with the membrane surface near N-terminus of the transmembrane alpha-helix. Dimerization mechanism of APP...
transmembrane domain has been described at atomic resolution for the first time that is important for understanding molecular events of APP sequential proteolytical cleavage resulting in amyloid beta-peptide.

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SW02.S7–98
Dimerization of transmembrane domain of human fibroblast growth factor receptor 3: implications for signaling and human pathologies
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Four human fibroblast growth factor receptors (FGFRs) belong to the family of receptor tyrosine kinases (RTKs) and transduce diverse biochemical signals by lateral dimerization in the plasma membrane. FGFRs play an important role in human growth and development, and in the adult. Among the family, FGFR3 is known for the largest number of pathogenic mutations observed in human. At least eight pathogenic mutations, implicated in cancers and growth disorders, have been identified in single-span transmembrane (TM) domain of FGFR3. Structural-dynamic properties of dimeric FGFR3 TM fragment L357-R399 were characterized by heteronuclear NMR spectroscopy in membrane-mimicking micellar environment. In solved NMR structure, two long TM helices (A369-L398) pack into a symmetric left-handed mimicking micellar environment. Inactivation of the dimer is characterized by heteronuclear NMR spectroscopy in membrane-stacking interactions of aromatic rings (Y379–Y382, S375–M378, A384–Y387, V371–L374). The central region of the dimer is characterized by intra- and intermolecular stacking interactions of aromatic rings (Y379–Y382), whereas the N-terminal part of the FGFR3 TM domain (A369–G372) becomes helical only upon dimer formation. Note-worthy, the most common pathogenic substitutions Gly380Arg and Ala391Glu causing the achondroplasia and Crouzon syndrome fall within the TM helix-helix interface. Nevertheless, several mutations are situated in weakly polar area rich in characteristic GG4-like motifs G370-xxG372, xxxS375-xxG378 and S377-xxG375 – a natural candidate for alternative dimerization mode of the FGFR3 TM helix. This implies that while the observed NMR structure is important for FGFR3 signaling, the mechanism of FGFR3-mediated transduction across the plasma membrane is complex and not yet understood. We propose a FGFR3 signaling mechanism that is based on the solved structure, the available structures of isolated soluble FGFR domains, and published biochemical and biophysical data. According to this mechanism the observed NMR structure corresponds to the basal phosphorylation receptor state, whereas the alternative dimeric conformation of the TM FGFR3 domain can be assigned to the fully active receptor.

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SW02.S7–99
Intramolecular distances in self-processing module studied by Trp-Trp energy transfer
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Self-processing module (SPM) is 177 amino acids-long segment of FrpC protein (Ferrum-regulated protein) produced by Neisseria meningitidis. FrpC possesses a unique calcium-dependent autocatalytic cleavage activity (Osicka et al., 2004) which is localized into its SPM module. However, the biological role and structure of the whole FrpC protein still remain unclear. The structural properties of SPM itself is difficult to determine due to its flexible loops (Macek, unpublished NMR data). Therefore, we employed various approaches (circular dichroism spectroscopy, NMR) in order to outline the structure of SPM. By employing NMR, we proved the existence of the flexible loop and also interaction between individual amino acids and the bound europium ion, a calcium analogue. By using CD spectroscopy, we found predominating exciton coupling peak (according to Ohmae et al., 2001) caused by the two close unique tryptophan residues W451 and W519 that shared their pi electrons. This approach, however, resulted only in partial characterization of SPM.

In case of SPM protein, both tryptophans and bound terbium ions (a luminiscent analogue of calcium) are in a close proximity. In order to find the distances between W451, W519 and bound calcium analogue, we studied the possible FRET between Trp/Trp and Trp/terbium. For this purpose, we used individual single tryptophan mutants of SPM (W451F, W519F) together with the wild-type SPM. By employing lifetime measurements and time-resolved fluorescence anisotropy of SPM tryptophans with/without terbium or calcium in frozen solution which eliminates depolarization caused by rotational motion of protein, we shed a light on the structural arrangement within a range of ~1 nm (resolution due to Foerster distance) inside SPM.

SW02.S7–100
Structural studies on DCL-1 (CD302), human leukocyte receptor
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DCL-1 is an eukaryotic receptor discovered few years ago as a fusion partner of C-type lectin receptor DEC-205 in a cell line derived from Hodgkin lymphoma [1].

Despite DCL-1 lacks single motives for carbohydrate binding in coordination with calcium cations it is classified as a C-type lectin according to its gene localization and sequence similarity. This receptor is predominantly expressed on phagocytic and antigen presenting cells, especially on monocytes and dendritic cells. There is a presumption, that DCL-1 plays a role in cell adhesion and migration. Another role of DCL-1 could be participation in endocytosis and subsequent targeting to lysosomes [2].

For our study we expressed a part of extracellular domain (D23 – R161), which included whole C-type lectin domain. Protein
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was produced in *E. coli* BL-21 (DE3) Gold in the form of inclusion bodies, *in vitro* refolded and purified by gel filtration.

For experiments of chemical cross-linking two homobifunctional (DSS, DSG) and one heterobifunctional (EDC) agents were used. Analysis of cross-linking reactions was made by high-resolution mass spectrometry.

For structure determination via NMR we produced $^{13}$C and $^{15}$N labeled protein. Stability and correct fold were confirmed by HN-HSQC experiment. For backbone resonance assignment HNCO and HNCACO (C$^\alpha$ assignment), HNCA and HN(CO)CA (C$^\beta$), HNCAB and CBCA(CO)NH (C$^\alpha$, C$^\beta$), (H)CC(CO)NH and HCCH-TOSCY (C$^\alpha$, C$^\beta$, side chain) spectra were analyzed. Hydrogen resonances were assigned using H(CC)(CO)NH, HN-TOSCY-HSQC and HCCH-TOSCY spectra. The motifs of secondary structure were predicted on the basis of backbone chemical shifts. The tertiary structure will be calculated on the basis of NOEY spectras.

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References

SW02.S7–102
Characterization of amyloid-beta oligomers and their elimination by D-enantiomeric peptides

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Alzheimer’s disease (AD) is a progressive neurodegenerative disorder affecting more than 35 million people worldwide. Extensive research studies have provided strong evidence for a central role of amyloid-$\beta$ peptide (A$\beta$) in the pathogenesis of AD implying A$\beta$ oligomers as the major toxic species. Consequently, the characterization of the oligomeric structure and the underlying aggregation mechanisms is essential for drug development.

In our work, we established a matrix-free assay based on density gradient centrifugation (DGC) for the preparation of homogeneous A$\beta$ oligomers, which allowed further characterization by different biophysical methods like atomic force microscopy (AFM) and circular dichroism.

We demonstrate, that the A$\beta$ oligomer is predominantly $\beta$-stranded, but do not show any detectable Thioflavin T binding, which is typical for the fibrillar structure. AFM analysis in aqueous, native-like environment revealed a diameter of 7.7 nm and a height of 4.5 nm for the oligomer, respectively. This oligomer is highly toxic to different types of differentiated SH-SY5Y human neuroblastoma cells.

Moreover, we established a method to quantify all kinds of A$\beta$ species like fibrils, oligomers and monomers, which are separated by DGC. This method enabled us an exact analysis of the A$\beta$ oligomer reduction by ligands and putative drugs. One of the most promising compounds in our studies was ‘D3’, a 12 D-amino acid-peptide selected by mirror-image phage display. In addition to A$\beta$ oligomer reduction D3 also reduced amyloid plaque load and cerebral damage of AD transgenic mice and improved their cognitive performance.

SW02 Biocatalytic Mechanisms and Protein Dynamics

A rational approach to improve the effectivity of D3 was to increase its local concentration. This was achieved by dimerization of D3. Indeed, the dimeric form of the ligand eliminates A$\beta$ oligomers much more effective than the double amount of the monomeric ligand.

We conclude that the combination of DGC and other biophysical techniques is very useful to prepare and characterize A$\beta$ oligomers and to screen for potential interactors, as shown for D3 and its dimeric form.

SW02.S7–102
Structure of C-terminal domain essential for folding of adenylate cyclase toxin from *Bordetella pertussis*

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Adenylate cyclase toxin (CyaA) of *Bordetella pertussis* is a member of Repeats-in-toxin (RTX) protein family of Gram negative bacteria. RTX proteins bind calcium ions by their nonapeptide glycine- and aspartate-rich repeats which subsequently create a typical secondary beta-roll structure which is crucial for folding and proper function of CyaA.

We produced, purified and crystallized C-terminal segment (CyaA 1529-1681) of CyaA containing about eight putative RTX motifs together with a C-terminal flanking region. In this region, a Block A with 15 amino acids was recently found to be crucial for an initiation of calcium-dependent folding of the whole CyaA molecule.

Escherichia coli BL21 was transformed with plasmid pET42b-CyaA1529-1681. The cyaA1529-1681 bore an N-terminal fusion with gene coding Glutathione-S-transferase and His Tag. Recombinant CyaA 1529-1681 was isolated by the two-step purification using Glutathione Sepharose and Nickel Sepharose columns, respectively. Gel filtration (TSKgel G2000SW) was used for further purification.

Commercial crystallization screens JCSG Core I-IV were used for CyaA 1529-1681crystallization employing the sitting-drop vapour-diffusion method. Crystals grown in three different conditions (containing polyethylene glycol 3350 or 2000 and inorganic salts or Tris-HCI) were used for X-ray analysis.

Diffraction datasets was collected with synchrotron in Helmholtz-Zentrum Berlin. Phase problem was solved by software Phaser-2.5.0 and for molecular replacement was used in silico created model of CyaA 1530-1680. Model of molecule was built in program Coot 0.6.2. and program Refmac 5.7.0029 was used for refinement.

Structure of CyaA 1529-1678 was solved for two crystals with space groups P 41212 and I 222 with resolution 1.45 Å and 1.23 Å, respectively. The final model binds eight calcium ions and forms regular beta-roll structure with atypical C-terminal anti-parallel beta-strands.
Conformity of local 3D geometry of protein molecules to tetrahedral water structure provides for over 90% discrimination between the native fold and the decoys

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We analyze inter-atom distance statistics for 3603 protein 3D molecular structures in a non-redundant subset of PDB (Hobohm, Sander, 1994). We show that the structure of water molecular networks around proteins is indistinguishable from that in the pure water, which implies that the biological macromolecules adjust their epitopic structure to the structure of water. Radial distribution functions (RDF) of water oxygen atoms around proteins show exactly the same peaks at the distances of about 2.76, 4.5, and 7 angstroms, and the peaks relative magnitude is also very similar to that in the pure water. An interesting feature of water density distribution in protein crystals is that there is a small but distinct peak in the oxygen atom RDF at 2.3 angstroms, which is experimentally observed in ice, but not in the liquid water (Head-Gordon, Hura, 2002).

We have designed an algorithm to computationally estimate the total free energy of solvation of a protein 3D molecular structure, using Monte-Carlo Reference State method for knowledge-based potentials for atomic interaction (Rakhmanov, Makeev, 2007).

We show that the local 3D geometry of soluble proteins, in terms of angles and distances in groups of polar atoms on the protein surface, is strongly optimized to accommodate the tetrahedral water structure. Furthermore, the conformity of local 3D geometry of protein molecules to tetrahedral water structure is a major factor in determining the native fold: it provides for over 90% discrimination between the native fold and the decoys in the case of improved Rosetta decoy set (Tsai et al, 2003). Thus, not only the general hydrophilicity of the protein atoms on the surface is essential in determining the free energy stabilizing the native fold, it is to a large extent the optimization of local 3D geometry of the polar groups on the protein surface that determines the protein folding and interaction.

Structural and functional characterization of the Staphylococcus aureus virulence factor and vaccine candidate FhuD2

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Staphylococcus aureus is a human pathogen causing globally significant morbidity and mortality. The development of antibiotic resistance in S. aureus highlights the need for a preventive vaccine. In the present paper we explore the structure and function of FhuD2 (ferric-hydroxamate uptake D2), a staphylococcal surface lipoprotein mediating iron uptake during invasive infection, recently described as a promising vaccine candidate. Differential scanning fluorimetry and calorimetry studies revealed that FhuD2 is stabilized by hydroxamate siderophores. The FhuD2–ferrichrome interaction was of nanomolar affinity in surface plasmon resonance experiments and fully iron(III)-dependent. We determined the X-ray crystallographic structure of ligand-bound FhuD2 at 1.9 Å (1 Å = 0.1 nm) resolution, revealing the bilobated fold of class III SBPs (solute-binding proteins). The ligand, ferrichrome, occupies a cleft between the FhuD2 N- and C-terminal lobes. Many FhuD2–siderophore interactions enable the specific recognition of ferrichrome. Biochemical data suggest that FhuD2 does not undergo significant conformational changes upon siderophore binding, supporting the hypothesis that the ligand-bound complex is essential for receptor engagement and uptake. Finally, immunizations with FhuD2 alone or FhuD2 formulated with hydroxamate siderophores were equally protective in a murine staphylococcal infection model, confirming the suitability and efficacy of apo-FhuD2 as a protective antigen, and suggesting that other class III SBPs might also be exploited as vaccine candidates.
death or apoptosis (1). At the structural level, Hrk is composed of an intrinsically disordered cytosolic domain containing the BH3 region and a C-terminal transmembrane domain responsible for its attachment to the membranes of intracellular organelles. Hrk’s interaction with prosurvival proteins like Bcl-xL and Bcl2 through its cytosolic BH3 region is key for the killing activity (2).

Structural information at atomic resolution on the free form of intrinsically disordered proteins (IDP) is scarce (3), most likely as a result of the difficulties associated to the presence of conformational heterogeneity. Within the Bcl-2 family, a recent example is found in the NMR study on the conformational behavior of the cytosolic domain of Hrk (4) and the factors involved in its interactions with other family members (5). However, additional biophysical factors can presumably play important roles when protein-protein interaction takes place in the membrane context. Information on this issue is critical to deepen our understanding on the molecular bases of apoptosis intrinsic pathway, as multiple Bcl-2 protein binding events occur in the mitochondrial membrane or in its proximities. Furthermore, structural studies on IDPs are essential to shed light into the ‘dark side’ of the structure-function paradigm (6).

Hrk is an ideal structure-function model that combines intrinsic disorder and membrane binding features. As a result of these uncommon characteristics, the expression and purification of Hrk poses significant challenges. In this work, we describe the expression and purification protocols for two different constructs of recombinant Hrk. One construct includes the cytosolic BH3 domain of the protein and will be used to perform NMR structural studies. The second construct is a truncated version of the full length protein containing the transmembrane domain, that will be studied in membrane mimetic experiments as a first step of the complete battery of biophysical studies.

References

SW02.S7–107
Structure and functional aspects of Rhodobacter sphaeroides Cryptochrome B
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Cryptochromes are blue light photoreceptors highly related to DNA photolyases. Together they form the photolyase/cryptochrome superfamily (pcsf). They are responsible for the maintenance of circadian rhythms in insects and animals and for a variety of photo responses in algae and higher plants. Despite these important functions in eukaryotes there are only few cyanobacterial cryptochromes known with assigned signalling functions [1].

Recently Cryptochrome B (RcCryB), a photoreceptor from Rhodobacter sphaeroides, was shown to interact with nucleic acids and to trigger responses to blue light and oxidative stress, e.g. gene repression [2]. RcCryB defines a separate clade of prokaryotic cryptochromes (crypro) inside the pcsf. Biochemical analyses showed a [4Fe-4S]-cluster in the C-terminal region of RcCryB, with a conserved binding motif in the crypro-family. The X-ray structure was solved at 2.7 Å resolution and showed a roof-like subdomain around the [4Fe-4S]-cluster which is required either for signalling or for structural stabilization of the nucleic acid binding cleft. Further analysis identified 6,7-dimethyl-8-ribityllumazine as novel antenna chromophore that is unique for the crypro-family. The binding moiety of the FAD chromophore has structural features which distinguish the crypro-family from the pcsf: (I) A water molecule interacts with the N5-nitrogen of the FAD and is important for the photochemistry of RcCryB. (II) Two tyrosines next to the FAD form the initial point of the photoreduction cascade, which takes a novel route via two tryptophans. Mutagenesis analysis showed that these two tryptophans are essential for photoreduction, in contrast to the tyrosines [3].

Through the structure and analysis of RcCryB we gathered deeper insights into the underlying molecular mechanisms of this novel photoreceptor. Especially the identification of the FeS-cluster sheds new light on the evolutionary relation between eukaryotic primases and members of the pcsf. Further studies focus on identifying possible RcCryB interaction partners and their relevance in vivo.

References

SW02.S7–108
Structural study of partially disordered delta subunit of RNA polymerase unique for gram-positive bacteria
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RNA polymerase (RNAP) from gram-positive bacteria such as Bacillus subtilis differs from well-studied RNAP of gram-negative bacteria in the presence of an additional subunit, δ. The presence of δ subunit increases the transcription specificity and the efficiency of RNA synthesis. Moreover, the absence of δ subunit is proposed to decrease the virulence of some pathogens and δ is required for competitive fitness of the cell. As crystallization at structure genomics centers failed, we focused on NMR studies of the δ subunit to describe its structure and internal dynamics. A previous study showed that the C-terminal domain of the δ subunit is unstructured and its repetitive sequence is highly acidic. Therefore, we started a systematic investigation of the protein with a shorter construct, corresponding to the well-structured N-terminal part and published its high-quality structure.

The full-length, partially disordered δ subunit of RNAP was studied by various NMR techniques. The structure of the well-folded N-terminal domain was confirmed based on inter-proton distances observed in the NOESY spectra. Heteronuclear steady-state nuclear Overhauser enhancement was employed to describe flexibility of the disordered C-terminal domain. Using paramagnetic labels, transient contacts of the C-terminal tail with the N-terminal domain and with itself were identified. A propensity of the C-terminal domain to form extended structures was obtained by the chemical shift analysis. The results proved that the δ subunit consists of a well-ordered N-terminal domain and a flexible C-terminal domain, which exhibits a complex hierarchy of partial ordering.

References
In summary, δ represents an excellent model protein to study molecules where the lack of structure does not mean a lack of function.

This work was supported by the Grants 204/09/0583 and 13-16842S from the Czech Science Foundation, by the 7FP of the EC (Contract 228461, EAST-NMR), and by the project ‘CEITEC – Central European Institute of Technology’ (CZ.1.05/1.1.00/02.0068) from European Regional Development Fund.

**SW02.S7–109**

H/Dex MS gives insight into RAGE receptor intra and intermolecular interactions

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The Receptor for Advanced Glycation Endproducts (RAGE) is a multiligand cell surface receptor. Activation of RAGE by its extracellular ligands plays an important role in certain human pathologies including diabetes, Alzheimer’s disease and cancer. However, it is still not well understood what are the specific mechanisms of recognition of different ligands by the receptor.

We will present H/D exchange MS studies for several extracellular subdomains of RAGE and its mutants under different experimental conditions and in the presence of ligands. Our data provide new insight into the molecular mechanism of activation of RAGE receptor.

**SW02.S7–110**

Oxidative protein folding pathway in mitochondria by NMR

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Disulfide bond formation is an essential step in oxidative folding and necessary for proteins to achieve their native conformation. Disulfide bonds formation occurs in most of cases in the subcellular compartments where the proteins functions and requires the coordinated action of specific proteins. Specifically an oxidative pathway in the mitochondrial intermembrane space is operative which involves the transfer of a disulfide from a specific protein to the substrate. This machinery involves the redox-regulated chaperone MIA40 and the sulphhydryl oxidase ALR. Proteins targeted to the intermembrane space are trapped by a disulfide exchange mechanism that involves an electron cascade from the incoming substrate to MIA40, then on to ALR, and finally to molecular oxygen via cytochrome c. Using the NMR spectroscopy, we found that the substrate protein is largely unfolded in the cytoplasm and that MIA40 in the IMS interact with a specific copy, we found that the substrate protein is largely unfolded in the cytoplasm and as a crucial recognition site in the disulfide relay compartments: it acts both as a mitochondrial targeting signal in the cytoplasm and as a crucial recognition site in the disulfide relay system of intermembrane space. A specific region of the N-terminal domain guides the interaction with the MIA40 substrate binding cleft (mimicking the interaction of the substrate itself).

The hydrophobicity-driven binding of this region ensures precise protein–protein recognition needed for an efficient electron transfer process. We dissected also the mechanism of the electron flux within ALR, characterizing at the atomic level the ALR intermediates that allow electrons to rapidly flow to cytochrome c.

**References**


**SW02.S7–111**

Characterization of two modes of 1,8-ANS binding to bacterial luciferase in viscous media by time-resolved fluorescent spectroscopy

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It is widely accepted now that in vivo conditions for enzymatic reactions are those of high viscosity caused by presence of both small and macro-molecules. The regulation mechanisms of enzymatic activity in such media are not entirely understood yet. The aim of the study was to determine the changes in structural properties of bacterial luciferase in viscous media of different nature. The binding sites of bacterial luciferase were investigated using the steady-state and time-resolved fluorescence of the probe 8-anilinonaphthalene-1-sulfonic acid (1,8-ANS). It’s believed that 1,8-ANS can interact with the proteins by hydrophobic or electrostatic mechanisms resulting in dramatically enhanced fluorescence of the probe.

Fluorescence of 1,8-ANS (1–200 μM) was monitored at a fixed concentration of *Photobacterium leiognathi* luciferase (2–8 μM) in phosphate buffer solution (0.05M, pH 6.9) and in the presence of glycerol and sucrose (40% w/v) using spectrofluorimeter Fluorolog 3-22 (Horiba Jobin Yvon) equipped with TCSPC. A downward-curved Scatchard plots were obtained from ANS emission at 470 nm indicating two types of binding sites for the probe. To define lifetimes the fluorescence decays of ANS were measured across the emission spectrum (with increment of 5 nm). The global analysis of the decays revealed presence of two types of emitters with short and long lifetimes (about 3 and 10 ns respectively) in all media. It can be result of the involvement of internal and external sites of luciferase molecule into binding of the probe. The photophysical characteristics for two types of fluorophores were obtained (spectral distribution, quantum yield etc.). Estimated contributions of each type of emitter into steady-state fluorescence intensity pointed out at the decreased affinity of the external binding sites of the luciferase molecule in viscous media.

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Remote control of protein–protein interactions: photo-switchable peptides for the regulation of clathrin-mediated endocytosis

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The possibility of remote monitoring and modulation of proteins activity from outside, triggering or inactivating them at a specific moment or in a defined location, is one of the new ambitious frontiers in chemical biology and molecular pharmacology [1]. Cellular function manipulation can be indirectly obtained using photo-controlled inhibitors or competitors of protein-protein interactions (PPIs), which are involved in almost every biological process [2]. Azobenzene has been successfully used as cross-linker to photo-regulate the folding of bio-active α-helices and eventually their specific functions. Following this strategy, we designed and synthesized photoswitchable inhibitors of protein-protein interactions (PPIPs), derived from the C-terminus region of β arrestin-2, to interfere with AP-2 adaptor complex, the essential hub in the interconnected network of clathrin-dependent endocytosis (CME) [3]. A Fluorescence Polarization based assay revealed the possibility of controlling and reversing the binding of our candidates to the desired protein target in vitro. Taking advantage of the spontaneous uptake, already shown by other ‘stapled’ peptides [4], we explored whether peptide activity could also be photo-controlled in living cells. The light-regulated internalization of transferrin receptor, monitored by flow cytometry and confocal microscopy, and the light-regulated modification of clathrin coated pits dynamics, assessed by total internal reflection microscopy (TIRFM), demonstrated our hypothesis. The selective manipulation of CME with light using these peptides, named Traffic Lights because they act as ‘stop’ and ‘go’ signals for membrane traffic, constitutes a novel tool to control cell signaling in spatiotemporally defined patterns. Traffic Light peptides can be used to dissect the role of CME in complex cellular functions.

References

Ligand binding and catalytic properties of cytochrome P450s from Mycobacterium tuberculosis

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Cytochrome P450s (P450) are likely involved in the main metabolic processes of mycobacteria and presumably participate in the degradation of most anti-tuberculosis (anti-TB) drugs. The variability of these enzymes may play an essential role in generation of new drug-resistant forms of mycobacteria.

In present work, we have performed molecular cloning, heterologous expression, isolation and purification of Mycobacterium tuberculosis P450s: P450 51 (MTCYP51), P450 124 (MTCYP124), P450 125 (MTCYP125) and P450 136 (MTCYP136). The substrate specificity of these enzymes was studied for various steroid derivatives, vitamins D and derivatives of methyl-branched lipids. We have shown that mycobacteria P450s are involved in biosynthesis/metabolism of both endogenous molecules (mycolic acids) and bioactive molecules of host organism. Also in our work a collection of substituted azole derivatives was used for screening of active site ligands and it was shown that some known antifungal drugs may have potential antimycobacterial activity.

In this work we have demonstrated for the first time data, confirming that binding of substrate and enzymatic reaction can be observed only near the physiological temperature of the pathogen host. This indicates on the specific dynamic characteristics of mycobacterial cytochrome P450s, which allow activating metabolomic processes of pathogen under certain conditions.

The study of P450s, with respect to differences in the structures, metabolic profiles and isozymes of resistant and nonresistant mycobacteria, may be a promising approach for anti-TB drug development and the treatment of tuberculosis. An understanding of the mechanism of P450s action would also aid in the elucidation of its function and role in pathogenesis of mycobacteria.

Thermodynamics of inhibitor binding to several recombinant carbonyl anhydrases isoforms

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Carbonic Anhydrases (CA) are a class of metalloenzymes present in both prokaryotes and eukaryotes. They contain a Zn(II) ion core, which is coordinated by three histidine imidazole groups and a single hydroxide ion, and form a distorted tetrahedral shape. CAs catalyse the reversible hydration of carbon dioxide [CO2 + H2O ↔ HCO3− + H+]. The CAs found in humans are the α-type. There are twelve active isoforms in humans (I, II, III, IV, Va, Vb, VI, VII, IX, XII, XIII, and XIV). They can be found in many parts of the cells including the cytosol, the cell membrane and the mitochondrion, as well as many tissue types including muscle, kidney, liver, etc. CAs are vital to life, however, overexpression of different CA isoforms has been linked to glaucoma, altitude sickness, obesity, and cancer. Design of selective CA inhibitors targeting a particular isozyme is important for pharmaceutical development.

Data will be presented for binding of several compounds designed by our group to the CA isoforms I, II, and XII. Thermodynamics of binding was determined by isothermal titration calorimetry (ITC) and thermal shift assays (TSA). TSA is a high throughput assay that measures protein stability dependence on added ligand concentration and can be used to find potential specific binding compounds. ITC gives the enthalpic and entropic contributions to the binding parameters. These parameters can be used to direct further development of new compounds with improved affinity and selectivity.
SW02.S7–115
Investigation of receptor activator of nuclear factor kappa B ligand and osteoprotegerin levels of obese and nonobese postmenopausal women
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Aim: The aim of the study was to investigate serum osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) levels and their correlations with bone mineral density (BMD) and levels of other bone turnover parameters such as serum osteocalcin, bone alkaline phosphatase (ALP), calcium, phosphorus and urinary deoxypyriridinoline (DPD) in obese and nonobese postmenopausal women.

Materials and Methods: Forty obese postmenopausal women [body mass index (BMI) >30 kg/m², 53.3 ± 5.1 years old] and 26 nonobese postmenopausal women (BMI between 18 and <24.9 kg/m², 55.9 ± 6.5 years old) were included in the study. Serum OPG, RANKL, bone ALP, osteocalcin, calcium, phosphorus and urinary DPD levels and BMD of all subjects were measured. Serum OPG, RANKL and osteocalcin levels were measured by ELISA method, serum bone-ALP levels were measured by chemiluminescent method, serum calcium and phosphorus levels were measured by routine methods and urinary DPD levels were measured by HPLC technique. The BMD was measured by dual-X-ray absorptiometry.

Findings: Serum RANKL (p = 0.006), RANKL/OPG ratio (p = 0.006), bone ALP (p = 0.028) and urinary DPD (p = 0.001) levels were significantly higher, whereas serum OPG (p = 0.015) levels were significantly lower in obese postmenopausal women than in nonobese postmenopausal women. There was no significant difference between serum osteocalcin, calcium and phosphorus levels and BMD measures of the groups. BMI was positively correlated with serum RANKL and urinary DPD levels in the obese group. Also, there was a significant negative correlation between age and femur BMD and between duration of menopause and femur BMD in the obese group.

Results: Increased RANKL levels and decreased OPG levels associated with osteoclastogenesis in postmenopausal obese women suggests that obesity plays a role towards osteoporosis in postmenopausal obese women. However, lack of any significant change in BMD levels of the subjects shows that the effect of increased RANKL/OPG ratio is compensated by increased bone formation which proved by increased bone ALP levels. OPG and RANKL levels are involved in the pathogenesis and regulation of bone turnover and thus circulating levels of them may be used as markers to investigate bone turnover and to develop new approaches to the evaluation of postmenopausal obese women.

SW02.S7–116
Significant variable sites and regions of hemoglobin beta-chains (HBB) aminoacid sequences of human and other primates
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The HBB aligned aminoacid (AA) sequences of 24 primates species (18 Haplorrhini and six Strepsirrhini) were analysed. It was found that this group of sequences has among 147 AA-position 45 variable sites with 72 AA-changes. Only 17 highly variable sites have two or more AA-changes while each of other 28 variable sites have only one AA-change. The location of protein variable sites and regions facilitates the graphical method (Garcia-Boronat M., Diez-Rivero C. et al., 2008).

Almost one half of variable sites HBB-molecules are located in three significant regions with 28 AA-position in common (1–14, 51–57 and 121–127). The variable sites of these three regions contain 37 AA-changes and the variability value is 1.32 per AA-position versus 0.49 value for the full length HBB-sequences. Only six of 17 variable sites with two or more AA-changes are located out of three variable regions.

Using great number of randomly generated homologous AA-sequences was possible to show that the presence of variable regions in HBB-molecules is not accidental and exceeds the stochastic ‘noise’. The N-terminal region 1–14 of homologous HBB-sequences is the most significant among three variable regions. But two other regions are significant only as satellites of the more variable region.

The most of 17 variable sites of HBB-molecules with two or more AA-changes are placed probably on the protein surface. The middle distance of side chains atoms of the appropriate non-Gly 15 AA-residues from human HBB-molecule center (18.7 Å calculated from X-ray data) is the evidence of this supposition. But this value for all 92 invariant position of aligned HBB-sequences is only 13.8 Å and it is evident that most of appropriate AA-residues are placed in the core of HBB-molecule.

Such ‘mutation carry information’ (Marx D.S., Colwell L.J. et al., 2011) from sequence alignment may be used to predict the protein folding.

SW02.S7–117
What makes [NiFeSe] hases special?
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Hydrogenases are metalloenzymes able to catalyze the reversible reaction \( H_2 \rightarrow 2H^+ + 2e^- \) at room temperature and without the use of overpotentials. In an era where \( H_2 \) is regarded as an alternative fuel, but there are no suitable catalysts for its oxidation or production in large scale, these enzymes are drawing increasing attention.

The [NiFeSe] hydrogenases are a subgroup of the [NiFe] family especially interesting due to their higher activities as well as higher tolerance towards to \( O_2 \) inhibition. In these hydrogenases one of the cysteines coordinating the nickel is replaced by a selenocysteine. The connection between the special properties of the [NiFeSe] hydrogenases and the presence of the selenocysteine is not clear, and there may be other factors that can contribute to this property.

In the enzymes of the [NiFe] class, the active site is deeply buried and therefore these enzymes need to have channels for \( H_2 \) diffusion as well as proton pathways connecting the active site and the exterior of the protein. Furthermore, these pathways have been shown to influence the catalytic properties of these enzymes.

In order to unveil the factors responsible for the special properties of [NiFeSe], we studied \( H_2 \) diffusion and proton pathways in [NiFeSe] hydrogenases using computational methodologies and compared them to the ones previously characterized in the standard [NiFe] hase from Desulfovibrio gigas. In the [NiFeSe] hydrogenases, we found an alternative channel that allows for the direct access of \( H_2 \) to the active site and is absent in standard [NiFe] hydrogenases, which could be related to the higher activities observed for [NiFeSe] hydrogenases. The proton transfer pathways of [NiFeSe] hases and [NiFe] hases are also different.

Reference

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SW02.S7–118
The effect of chemical cross-linking on protein structure and function
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Chemical cross-linking coupled with mass spectrometry is a powerful technique for characterization of the architecture of proteins and protein complexes. In chemical cross-linking, the residues which are in close proximity to each other may be converted into covalent bond by cross-linker. The cross-linking residues which are then efficiently identified by mass spectrometry provide experimental distance constraints which may be applied for modeling purposes.

In recent years, the popularity of cross-linking has expanded and significant progress has been made in applications and bioinformatics improvements. However, the question if chemical cross-linking distorts the protein structure has not been investigated so far. In the present study, we have looked into the effect of the cross-linking on the structural and functional properties of human carbonic anhydrase (hCA-I).

This enzyme was cross-linked with the homobifunctional cross-linkers BS3 and BS2G. After cross-linking, measurements of enzyme activity was carried out under various conditions, such as cross-linker and enzyme concentration. Furthermore, the cross-linked enzyme was analyzed by SDS PAGE electrophoresis. In order to gain a more detailed insight into the structure of cross-linked enzyme, 15N labeled hCA-I was upon cross-linking analyzed using NMR spectroscopy by measuring of the 1H-15N TROSY spectra which were compared with the spectrum of unmodified enzyme.

Measuring of enzyme activity of cross-linked hCA-I revealed significant influence of cross-linking on enzyme activity. The chemical shift changes of the backbone amide were monitored by measuring of the 1H-15N TROSY. Superposition of the 1H-15N TROSY spectra of the unmodified and cross-linked hCA-I revealed that the relative intensities and the peak positions of several well-resolved peaks were significantly different, whereas interestingly a large majority of cross-peaks were rather slightly affected. The overall chemical shift perturbation pattern caused by BS3 is different to BS2G which confirm their different effect on protein structure, however the NMR data indicate that cross-linking of hCA-I has little effect on the protein structure.

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SW02.S8 Protein Dynamics (II-W8)

SW02.S8–1
Dynamic mechanisms of inhibiting protein–protein interactions in translation initiation for design of anti-tumor agents
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Eukaryotic translation is regulated by features of the 5′UTR and by the concentrations and state of initiation factors. mRNAs with short 5′UTR are translated efficiently, do not rely heavily on a functional initiation machinery and are thus called strong mRNAs. Weak mRNAs have long 5′UTRs, are translated poorly and rely on a strong initiation complex. Weak mRNAs code predominantly for oncogenes, growth factors and anti-apoptotic proteins, which are thus down regulated by the nature of their 5′ UTR. Among the initiation factors, the concentration of the cap-binding protein eIF4E seems to be rate limiting. The eIF4E protein recruits the small ribosomal subunit to the 5′ end of the mRNA via interactions with eIF4G and eIF3. Elevated concentrations of eIF4E have been found in several forms of cancer. The activity of eIF4E is regulated by the 4E-binding proteins (4EBPs), which are targets of kinase in signaling pathways and are validated tumor suppressors. Other factors, such as eIF1, eIF1A, eIF5 and eIF2 regulate start-codon recognition.

Through structural and functional studies we try to understand the complex mechanism of translation initiation that are responsible for cellular homeostasis, cellular transformation in cancer and cancer stem cells due to loss of translational balance.

We hypothesized that weakening the interaction of eIF4E with eIF4G would selectively reduce the translation of oncogenes and messages for growth-promoting proteins. Using high-throughput screening we discovered small molecules that inhibit the eIF4E/eIF4G interaction. The inhibitors termed 4EGI displaces eIF4G from eIF4E but stabilize the interaction with 4EIBP-1. Binding of the initial hit compounds and analogs to eIF4E was studied with NMR, X-ray crystallography and other biophysical techniques. The compounds and analogs were tested in in-vitro and in-vivo assays. The molecules exhibit activity against melanoma, breast, lung, prostate cancer and acute myelogenous leukemia (AML). The lead compounds inhibit eIF4E/eIF4G interactions in xenograft tumors in mice and reduce tumor growth. 4EGI-1 inhibits tumor expression of oncogenic proteins such as cyclin E, cyclin D1, c-myc and Bcl-2. Intra-peritoneal treatment with 4EGI-1 did not exhibit any toxic effects in mice. Recently we determined high-resolution structures of eIF4E/inhibitor complexes and discovered that the inhibitors act by an allosteric mechanism. We also obtained structures of the eIF4E complex with a large fragment of 4EIBP-1, which revealed the mechanism of stabilization by 4EGI1.

SW02.S8–2
Structure and dynamics in Lac repressor-DNA interactions
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The E. coli lac operon is the classical model for gene regulation in bacteria. An overview will be given of our work on the lac repressor-operator system. An early result was the 3D structure of lac headpiece in 1985, one of the first protein structures determined by NMR. Our studies of the structure and dynamics of complexes of a dimeric headpiece construct with lac operator DNA have provided a detailed picture of how the various lac operator sequences are recognized. Furthermore, comparison with the non-specific DNA complex clarified how the repressor searches for its target site by sliding along random DNA and binds to the operator through a folding-coupled-to-binding transition. Surprisingly, the 1D diffusion rate obtained from NMR is much slower than that determined by single molecule fluorescence methods and cannot account for an enhanced target location by lac repressor.

We have also investigated the mechanism of allosteric coupling of the lac repressor. As all allosteric changes occur in the dimer we use a dimeric form of lac repressor (70 kD), which lacks the tetramerization domain. From 15N chemical shifts of the inducer (IPTG) bound and operator bound complexes we could deduce the allosteric mechanism. Furthermore, the ternary complex with both inducer and DNA bound could be characterized. Some of the results are different from what the crystal structures suggest.
Dynamics of substrate-induced conformational changes in determining enzyme specificity
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Nearly every enzyme shows a change in structure occurring with substrate binding, but the pathway and role of these substrate-induced conformational changes in enzyme specificity has been controversial. DNA polymerases provide an ideal model system to explore enzyme specificity since the alternative substrates are well known and the template-dependent polymerization allows single-turnover kinetic studies to be performed. Here we explore the role of nucleotide-induced conformational changes using HIV reverse transcriptase. Addition of a fluorescent label to the enzyme provided a single to monitor the kinetics of the substrate-induced enzyme conformational change. We show that initial weak nucleotide binding \( K_d = 200 \text{ } \mu\text{M} \) induces a fast transition from the open to the closed state (2000/s), but re-opening of the enzyme is slow (\(~1/\text{s}\) relative to chemistry (\(~30/\text{s}\)). Thus, specificity is solely determined by the initial weak binding and the rate of the fast conformational change. We have also explored the molecular details governing the initial weak nucleotide binding and fast enzyme conformational change by molecular dynamics (MD) simulations using the Milestoning methods. The simulations and experimental data support an induced-fit model and are inconsistent with models invoking capture of the nucleotide by the closed state. We show that the transition from open to closed enzyme conformation is fast in the presence and absence of the correct substrate. However, only the correct substrate leads to a rapid, thermodynamically favored collapse to the final closed state from a transition state where all charged residues are aligned and within 10 A of the substrate. Mismatched nucleotides fail to align properly at the active site and do not stabilize the closed state. MD simulations predict rates that are consistent with experimental observations.

Structure and functioning of copper chaperones
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Copper chaperones compose a specific class of proteins assuring safe handling and specific delivery of potentially harmful copper ions to variety of essential copper proteins like Cu-ATPases, Cu, Zn-superoxide dismutase (SOD) and cytochrome c oxidase. Metallation of Cu-ATP-ases is performed with copper efflux chaperone Atx1 (yeast) or Hah1 (human). Cu, Zn-SOD is metallated with copper chaperone for SOD – Ccs. Metallation of cytochrome c oxidase is apparently the most complicated task of copper delivery as it requires highest number of assisting proteins, such as Cox11, Cox17, Sco1, Sco2, Cox19 and Cox23. Copper chaperones compose structurally heterogeneous class of proteins, which can exist in multiple metal-loaded as well as oligomeric forms. Moreover, many copper chaperones (Ccs, Cox17, Sco1) exist in various oxidative states and participate in redox catalysis, connected with their functioning. Analysis of the structural and functional properties copper chaperones and their partners allowed us to define specific regulatory principle in copper metabolism connected with copper-induced conformational control of copper proteins.

Conformational dynamics of DNA repair enzymes revealed by fluorescence stopped-flow analysis
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Many forms of DNA base damages arise in cells and only the continuous action of specialized DNA repair systems can prevent a rapid decay of genetic information. Single-base lesions are eliminated by base excision repair (BER), a pathway initiated by DNA glycosylases that recognize and excise damaged bases.

DNA glycosylases are faced with the difficult task of recognizing their substrates in a large excess of unmodified DNA. Structural data suggest that conformations of DNA glycosylases and their substrate DNA are often changed in the ES complex in comparison with the free enzyme and substrate molecules. Therefore, it can be reasonably suggested that these conformational changes are responsible for the specificity of BER enzymes.

In our works, we have used stopped-flow kinetic approach to study the conformational dynamics of 8-oxoguanine-DNA glycosylases, Fpg protein from E. coli and human OGG1, during lesion discrimination steps. These enzymes belong to the class of DNA glycosylases/absic site lyases. The intrinsic Trp fluorescence in proteins and 2-aminopurine (2-aPu) or pyrrolocytosine (pyrC) fluoroses as well as FRET for donor/acceptor pair in DNA containing damaged sites were recorded.

Multiple transient changes in fluorescence intensities of enzymes and DNA substrates indicate sequential conformational changes in both macromolecules during the catalytic cycles. Thermodynamic parameters of each recognition step were found by analysis of fluorescence traces at different temperatures. Combining these data with the structural information available for enzyme-DNA complexes, we have been able to describe the most likely sequence of events leading to the preferential recognition of lesions by repair enzymes and discrimination against other possible substrates.

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Recognition of DNA damages by human 8-oxoguanine DNA glycosylase
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Extensive structural studies of human DNA glycosylase hOGG1 have revealed the substantial conformational changes of enzyme molecule; however, at present there is little information about the time scale of rearrangements of protein structure as well as dynamic behavior of individual amino acids. Here we examined the role of certain catalytically important amino acids in hOGG1 enzymatic pathway and described their involvement in the step-by-step mechanism of oxidative DNA lesion recognition. The
conformational dynamics of hOGG1 wild-type and mutants Y203W, Y203A, H270W, F45W, F319W and K249Q and DNA-substrates were investigated by the fluorescence stopped-flow method.

The analysis of kinetic data obtained in this study significantly improves understanding of the step-by-step molecular mechanism of hOGG1 lesion recognition process. Our data showed that the function of Tyr-203 residue is not only to conserve of the kinked state of DNA duplex but to serve as ‘lesion-sensor needle’ in the discrimination between normal and damaged bases. Also we demonstrate that Lys-249 plays an important role in the early step of the damaged nucleotide binding and flipping out process. The suggestion that Asp-268 residue is responsible for hydrolysis of N-glycosidic bond, whereas Lys-249 is a key amino acid in sugar-phosphate bond cleavage ($\beta$-elimination reaction) was confirmed. The role of His-270 in the oxoG flipping out process was identified.

This study was made possible by grants from the Program of the Russian Academy of Sciences ‘Molecular & Cell Biology’ (6.11), RFBR (13-04-00013 and 12-04-31066) and Russian Ministry of Education and Science (SS-64.2012.4, SP-4012.2013.4, 8092, 8473 and 14.B37.21.0195), the Grant from Russian Government to support leading scientists (No. 11.G34.31.0045) to N.A.K., A.A.K. and O.S.F, as well as the grants [ANR Blanc 2010 Project ANR- 09-GENO-000, PICS N5479, RB 2013] to M.K.S. and [a2012 00029161] to A.A.I.

Abstracts

**SW02 Biocatalytic Mechanisms and Protein Dynamics**

**SW02.S8–7**

Dynamics of the signal transducer protein HemAT as revealed by time-resolved step scan FTIR spectroscopy

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HemAT is a heme-based O$_2$ sensor protein that acts as a signal transducer for aerotaxis in *Bacillus subtilis*. In our work, we have employed time-resolved step-scan Fourier transform infrared (TRS$^2$-FTIR) spectroscopy to investigate the protein structural changes induced by ligand (CO) photodissociation and rebinding that are crucial for understanding the initial events of the intramolecular signal transduction mechanism in HemAT. We have studied the full length and truncated sensor domain HemAT- CO adducts as well as the Y70F, L92A, T95A and Y133F mutants. Monitoring the rebinding of CO to the heme-Fe$^{2+}$ reveals biphasic kinetics for both the full length and truncated sensor domain HemAT. The TRS$^2$-FTIR experiments demonstrate that Y70 affects the conformational changes that are induced to the protein matrix by CO photodissociation. Moreover, L92 appears to operate as the conformational gate in the migration pathway of photodissociated CO.

**SW02.S8–8**

Protein conformational dynamics in the mechanism of HIV-1 protease catalsis

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Total chemical synthesis was used to prepare a series of unique chemical analogues of HIV-1 protease (HIV-1 PR) [1], where we systematically substituted the residues Gly51, Gly51’ at the tips of the mobile ‘flaps’ (residues 37-61 in each domain of the protein homodimer) with L-Ala, D-Ala in both symmetric and asymmetric fashion. Such substitutions, although in regions distant from the catalytic aspartates, led in most cases to reduction of catalytic activity. In contrast to this, a ‘covalent dimer’ with L-Ala51 in one flap and D-Ala51’ in another flap has shown native-like enzyme activity. To gain insight into molecular details of such results we applied a variety of biophysical methods, including NMR and pulsed-EPR spectroscopies, as well as X-ray crystallography, MD simulations and appropriate enzymatic assays [2,3]. We showed that conformational isomerizations in the flaps are correlated with structural reorganization of residues in the active site, and it is reorganization of the active site that is a rate-limiting factor in catalysis of HIV-1 protease. The asymmetric conformational dynamics in the [D-Ala51, L-Ala51’] HIV-1 analog enzyme leads to conformational states of the catalytic apparatus in which residues Asp$^{2+}$ and Asp$^{2+}$ and the nucleophilic water molecule are prearranged for catalysis, further emphasizing the complexity of interdomain communication in the HIV-1 protease homodimer protein molecule.

References


**SW02.S8–9**

The high-affinity inorganic phosphate transport system of *Saccharomyces cerevisiae*: a tale of two proteins

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Acquisition of nutrients from the surrounding environment is a prerequisite for cell survival. The nutrient inorganic phosphate is no exception on this rule. Even more so, inorganic phosphate plays a role in numerous metabolic pathways. Yet, in order for a cell to accumulate sufficient amounts of inorganic phosphate, several transporters systems are needed to accomplish this goal. In *Saccharomyces cerevisiae* two inorganic phosphate transport systems are responsible for the accumulation of inorganic phosphate. During conditions where there is an ample amount of inorganic phosphate in the environment, a low-affinity system consisting out of three transporters (Pho87, Pho90 and Pho91) is operational. During conditions where there is a lower available inorganic phosphate, the high-affinity system will be operational. This high-affinity system consists out of two transporter proteins, being Pho84 and Pho89. Arguably, Pho84 is the most study of the two, with its cellular function being well understood. Yet, the underlying molecular mechanisms involved in substrate transport are still largely unknown. We’ve recently applied a rational approach based on multiple sequence alignments and an *in silico* generated model of Pho84 to design several site-directed mutants. The analysis of these mutant alleles has broadened our knowledge concerning the putative phosphate binding site and the nature of the Pho84 transceptor functionality. In contrast to the up to date knowledge about the Pho84, the Pho89 high-affinity phosphate transporter has been much less studied. Yet, being member of the PiT (SLC20) family (to which the human hPiT-1 and hPiT-2 belong), Pho89 can serve as an excellent candidate to study. We have recently investigated the functional importance of the Pho84, the Pho89 high-affinity phosphate binding and flipping out process.

References

**Role of NHERF2 adaptor protein in endothelial cells**

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Adaptor proteins mostly function as flexible molecular scaffolds and tend to lack enzymatic activity but instead mediate specific protein-protein interactions and facilitate the formation of larger signaling complexes. NHERF2 (Na+/H+ exchanger regulatory factor 2) and EBP50 (ezrin-radixin-moesin (ERM)-binding phosphoprotein 50) are ERM binding domain containing adaptor proteins. Previously we found unusual nuclear localization of EBP50 in endothelial cells (EC) and cell cycle dependent specific interaction was detected between EBP50 and protein phosphatase 2A. The aim of the present study was exploring the role of NHERF2 adaptor protein in EC.

Our results suggest that in EC NHERF2 and EBP50 adaptor proteins have different binding affinity towards the individual ERMs. It was verified by pull down assays, immunoprecipitations and by overexpression of recombinant proteins. During mitosis, phospho-ERMs seem to bind to NHERF2 in the plasma membrane. Phosphorylation level of ERMs increased by overexpressing wt NHERF2, but not mutant NHERF2, that missed the ERM-binding tail. Furthermore, in NHERF2 silenced cells, the phosphorylation level of ERMs was much lower, compared to wt NHERF2, but not mutant NHERF2, that missed the membrane. Phosphorylation level of ERMs increased by overexpression of recombinant proteins. During mitosis, phospho-ERMs seem to bind to NHERF2 in the plasma membrane. Phosphorylation level of ERMs increased by overexpression of recombinant proteins. During mitosis, phospho-ERMs seem to bind to NHERF2 in the plasma membrane.

Our results also indicate that NHERF2 can modulate the phosphorylation level of ERMs.

**Effect of apoA-I mutations in the capacity of reconstituted HDL to promote ABCG1-mediated cholesterol efflux**

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ATP binding cassette transporter G1 (ABCG1) mediates the cholesterol transport from cells to high-density lipoprotein (HDL), but the role of apolipoprotein A-I (apoA-I), the main protein constituent of HDL, in the capacity of HDL to promote ABCG1-mediated cholesterol efflux has not been examined. Here we measured cholesterol efflux from HEK293 cells or J774 mouse macrophages overexpressing ABCG1 using as acceptors reconstituted HDL (rHDL) containing wild-type or various mutant apoA-I forms. ABCG1-mediated cholesterol efflux was found to be severely decreased (by 89%) by the carboxyl-terminal deletion mutant apoA-I[D(185–243)]. ABCG1-mediated cholesterol efflux was not affected or moderately decreased by amino-terminal deletion mutants and several mid-region deletion or point apoA-I mutants, and was restored to 69–99% of control by double deletion mutants apoA-I[D(1–41D)(185–243)] and apoA-I[D(1–59D)(185–243)]. These findings suggest that the central helices alone of apoA-I associated to rHDL can promote ABCG1-mediated cholesterol efflux. Further analysis showed that the carboxyl-terminal deletion mutant apoA-I[D(185–243)] only slightly reduced (by 22%) the ABCG1-mediated efflux of 7-ketocholesterol, indicating that, depending on the sterol type, structural changes in rHDL-associated apoA-I affect differently the ABCG1-mediated efflux of cholesterol and 7-ketocholesterol. Overall, our findings demonstrate that rHDL-associated apoA-I structural changes affect the capacity of rHDL to accept cellular cholesterol by an ABCG1-mediated process. The structure-function relationship seen here for rHDL-associated apoA-I mutants and ABCG1-mediated cholesterol efflux closely resembles results obtained for lipid-free apoA-I mutants and ABCA1-dependent cholesterol efflux, suggesting that both processes are dependent on the same structural determinants in apoA-I.

**Regulation of protein synthesis pattern in skeletal muscle under early stage of hindlimb unloading**

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Skeletal muscle atrophy occurs during gravitational unloading or hypokinesia [Thomason et al., 1990; Flück et al., 2002]. Postural skeletal muscles, soleus and gastrocnemius, suffer severe from atrophy under wasting which results from an imbalance between rates of protein synthesis and breakdown. Loss of the skeletal muscle mass happens as early as 3 days hindlimb unloading [Lomonosova et al., 2011]. Activation of proteolytic enzymes namely calpains and E3-ligases, MuRF1 and MAFbx, can take part in proteolysis during the unloading [Bodine et al., 2001]. Moreover, protein synthesis decrease was found as early as 3 days hindlimb suspension [Loughna et al., 1986]. The aim of our study was analysis of protein synthesis pattern in rat soleus in conditions of short-term hindlimb unloading. Male Wistar rats were divided into two groups: control group (C group, 221–225 g, n = 10), hindlimb unloaded group during 3 days (3HS group, 226–240 g, n = 8). Hindlimb unloading was carried out according to Novikov-Ilyin’s technique with Morey-Holton’s modification. The rats were sacrificed by nembutal overdose (75 mg/kg body wt), each soleus was weighted, immediately frozen in liquid nitrogen and stored at −80°C until analysis. Atrophy of soleus was found in the hindlimb unloaded group. Total and phosphorylated levels of p70S6-kinase lying on signaling pathway downstream of mTOR and regulating protein translation by phosphorylating ribosomal protein S6 [Adegoke et al., 2012] remained unchanged in skeletal muscle during short-term functional unloading. Eukaryotic elongation factor 2 (eEF2) is one of the important member of eEF family catalyzing simultaneous translocation tRNA and mRNA on a 80S ribosome [Taylor et al., 2007]. It’s known that eEF2 phosphorylation by its specific eEF2-kinase leads to prevention of elongation translation [Browne et al., 2002]. We observed two-fold increase of eEF2k mRNA expression in rat soleus as early as 3 days unloading as compared to control group (p < 0.01). P-eEF2 level was also increased significantly after 3 days unloading in comparison with the control group (p < 0.05). When analyzing eEF2 mRNA expression it wasn’t found any changes in its level after the unloading. There were not any changes in expression of 18S ribosomal RNA and ribosomal protein L19 mRNA. It is concluded that increase in eEF2 phosphorylation is sufficiently associated with the transcriptional regulation of eEF2-kinase in rat soleus after short-term hindlimb unloading.

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Mouse nuclear myosin I knock-out shows interchangeability and redundancy of myosin isoforms in the cell nucleus

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Nuclear myosin I is a nuclear isoform of the well-known 'cytoplasmic' Myosin 1c protein. Located on the 11th chromosome in mice, NM1 results from the alternative start of transcription of the Myo1c gene adding an extra 16 amino acids at the N-terminus [1]. Previous studies revealed its roles in RNA Pol I and RNA Pol II transcription [2, 3], chromatin remodeling [4], and chromosomal movements [5]. It was thought, that nuclear localization signal is localized within first 16 amino acids. However, we discovered that is localized in the middle of the myosin molecule and therefore directs both Myosin 1c isoforms to the nucleus. In order to trace specific functions of the NM1 isoform, we generated mice lacking the NM1 start codon without affecting the cytoplasmic Myo1c protein. Mutant mice were analyzed in a comprehensive phenotypic screen and strikingly, no obvious phenotype related to previously described functions has been observed. However, we found minor changes in bone mineral density and the number and size of red blood cells in knock-out mice, which are related to previously undocumented functions of NM1 in the cytoplasm. Moreover, in Myo1c/NM1 depleted U2OS cells, the level of Pol I transcription was restored by overexpression of shRNA-resistant mouse Myo1c. The ratio between Myo1c and NM1 proteins is similar in the nucleus and deletion of NM1 did not cause any compensatory overexpression of Myo1c protein. Finally, we found that Myo1c is in interaction with Pol II. We therefore suggest that both isoforms can substitute each other in their nuclear functions.

References

A protein kinase (MKK6) can also function as a phosphatase

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Protein kinases are essential components of signal transduction. In eukaryotic cells there are a number of coexisting MAP kinase pathways, which each comprise a series of three kinases which sequentially phosphorylate and activate the next downstream kinase. These pathways respond to different stimuli and elicit different cellular responses. Protein phosphatases function as negative regulators that switch off the pathways when required. Protein kinases generally possess a common catalytic architecture and mechanism, utilising a general acid/base residue (normally aspartate) and positively charge sidechains and metal cations to counterbalance the negatively charged phosphoryl group. On the other hand they must also have specificity for phosphorylating specific residues of a particular substrate, particularly in the case of the parallel MAP kinase pathways.

Our work has shown that a dual specificity MAP kinase kinase (MKK6) can also act as a phosphatase towards its substrate kinase (p38a). The discovery of phosphatase activity in MKK6 is a surprising and novel finding, since this activity has not previously been observed for any protein kinase. The mechanism of this activity is Mg2+ dependent and requires a catalytic aspartate residue that performs the role of general acid/base; this illustrates that MKK6 uses the same active site for both kinase and phosphatase activities. It is likely that other protein kinases also possess a secondary phosphatase activity, due to the common nature of the catalytic site for both processes, which has not yet been observed.

The phosphatase activity of MKK6 occurs on a slower timescale than its kinase activity and is favoured in the absence of nucleotide. It is found to have a preference for dephosphorylating tyrosine over threonine. Furthermore its phosphase activity is significantly enhanced by its own activation by its upstream kinase. In addition, MKK6 is found to be capable of phosphorylating residues other than the canonical p38a phosphorylation sites, for example in autophosphorylation. This non-specific kinase activity may compromise specificity and so it may be preferable to reverse unwanted phosphorylation events.

Therefore phosphatase activity of a protein kinase may provide evolutionary advantages for an organism. Firstly it may provide an alternative means of negatively regulating signalling pathways mediated by kinases, without the need for a separate phosphatase. Secondly it may serve as a ‘proof-reading’ function that helps to confer specificity of kinases towards phosphorylating particular residues of a target substrate.

Anion-transport inhibitors interact and inhibit VDAC1 oligomerization

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Apoptotic signalling to the mitochondria results in the release of pro-apoptotic proteins, such as cytochrome c (Cyto c), from the intermembranal space to the cytosol, triggering caspases activation and cell destruction. However, the mechanisms by which apoptotic initiators, including Cyto c, are released from mitochondria remain unclear. Accumulating evidence indicates that the voltage-dependent anion channel 1 (VDAC1), an outer mitochondrial membrane protein, plays a central role in mitochondria-mediated apoptosis. In a previous study we have
demonstrated that apoptosis induction is associated with VDAC1 oligomerization, and suggested that VDAC1 oligomer forms a mega pore mediating the release of Cyto c.

In this study, we further support the involvement of VDAC1 oligomerization in apoptosis. We tested the effects of known anion-transport inhibitors, DIDS, H2DIDS, SITS, DNDS and DPC, on VDAC1 oligomerization and apoptosis. These reagents are able to interact directly with VDAC1 and prevented both VDAC1 oligomerization and apoptosis, as induced in HeLa cells by various apoptosis inducers. VDAC1 oligomerization in intact cells was revealed by chemical cross-linking, while apoptosis was determined by FACS analysis. The results indicate VDAC1 to be a component of the apoptosis machinery and support the suggestion that VDAC1 oligomerization is coupled to apoptosis induction. Targeting the VDAC1 oligomeric status, and hence apoptosis, thus offers a therapeutic strategy for neurodegenerative diseases such as Alzheimer or Parkinson’s disease.

**SW02.S8–16**

**From whole cells towards photosynthetic reaction centres: dynamics properties for biotechnological applications**

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Photosynthesis gain renewed interest due to the possibility to integrate whole plant cells or their photosynthetic sub-components into optoelectronic devices such as biosensors for environmental monitoring. In this context, it is of great relevance to study the function/dynamics relationships of genetically modified photosynthetic organisms, in order to identify the parameters underlying an increased performance in terms of charge separation, protein stability and functional reliability. Here, we address the question if there is a ‘functional’ dynamics in addition to the intrinsic dynamical behaviour common to all proteins and how do they couple. In particular, understanding if ‘rigidity’ is essential for the charge transfer process and if this property is shared by all the photosynthetic systems and how this information can be apply to design high performant bio-sensors. To this end a study of reaction centres: dynamics properties for biotechnological applications undertook using neutron scattering experiment. Some of these components into optoelectronic devices such as biosensors for environmental monitoring.

**References**


**SW02.S8–18**

**ESR-PELDOR studies of structural transitions of DNA induced by DNA repair enzyme**

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The goal of this study was to identify the structural perturbation of the DNA in the sites of lesion location and to realize how these features are crucial for enzyme binding. pulsed electron-electron double resonance (PELDOR) spectroscopy was applied to determine spin–spin distances in spin-labeled DNA duplexes (13-mer and 17-mer) containing damaged sites 8-oxoguanine or uncleavable abasic site analogue tetrahydrofuran. The lesions were located in one strand of the DNA, and two nitroxyl spin labels were attached at the 5′- and 3′- ends of the complementary strand. PELDOR data indicate on decrease of the distance between the two spin labels in damaged DNA, which was interpreted as a result of the bending of the DNA duplex induced by the lesions. Results of PELDOR measurements were supported by molecular dynamic calculations. Interaction of DNA fragments with DNA repair enzyme 8-oxoguanine-DNA glycosylase from E. coli (Fpg protein) was also studied. PELDOR data indicate that this interaction leads to a further significant decrease of the distance between spin labels, which evidences the enzyme-induced extra bending of the DNA duplex. The results may appear to be useful for studying mechanisms of fast searching and initial recognition of damaged sites by DNA repair enzymes.

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**Analysis of the interaction between gastrokin1 and amyloid-beta peptide: a potential anti-amyloid activity of the protein**

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**Aim:** Gastrokin1 (GKN1) is a stomach-specific protein important for maintaining the physiological function of the gastric mucosa. GKN1 is characterized by the presence of a BRICHOS domain consisting of about 100 amino acids found in several unrelated proteins associated with major human diseases (Sánchez-Pulido et al., 2002) like BR12, related to familial British and Danish dementia; chondromodulin-I (CHM-I), linked to chondrosarcoma; surfactant protein C (SP-C), associated with respiratory distress syndrome; and gastrokines (Shiozaki et al., 2001; Martin et al., 2003), linked to gastric cancer. Literature data show that recombinant BRICHOS domains from Br12 and SP-C precursor (proSP-C) prevent fibril formation of amyloid-beta peptide (Aβ) that is the major component of extracellular amyloid deposits in Alzheimer’s disease (Willander et al., 2012). Aβ derives from the partial hydrolysis of the amyloid precursor protein (APP) catalyzed by β- and-γ-secre-tase. The hydrolysis produces amyloid peptides of 40 or 42 amino acid residues. Here we investigate the interaction between recombinant GKN1 (rGKN1) (Pavone et al., 2013) and Aβ (1-40).

**Methods:** Aβ was incubated in presence or absence of human rGKN1 and chicken cystatin, as negative control, at 5:1 molar ratios. Samples were then analyzed by SDS-PAGE. The aggregation of Aβ was also evaluated using Thioflavine T binding assay. Blue Native Page (BN-PAGE), BIAcore technique, isothermal titration calorimetry (ITC) and mass spectrometry were performed to characterize the interaction.

**Results:** GKN1 prevented amyloid aggregation and fibril formation by inhibiting Aβ (1-40) polymerisation. This result was also confirmed by Thioflavine T binding experiments. BN-PAGE, ITC and mass spectrometry showed the formation of rGKN1/Aβ complex. BIAcore kinetics of rGKN1/Aβ interaction led to calculate a dissociation constant (KD) of 34 μM.

**Conclusions:** These preliminary data strongly indicated that GKN1 poses anti-amyloid activity thus, it might play a role of chaperone directed against unfolded segments with an ability to recognize amyloidogenic polypeptides and prevent their aggregation.

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**Mechanism of ATP hydrolysis by the archaeal TIP49 AAA+ protein**

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Eukaryotic TIP49a and TIP49b belong to the AAA+ superfamily and form oligomers that are found in multi-protein complexes involved in chromatin remodeling, DNA repair, telomerase and ribonucleoprotein particle assembly. These proteins play essential but yet unknown role(s) as chaperones or adaptor proteins and are implicated in gene expression, ribosomal RNA processing, apoptosis, cell division and proliferation.

We used the TIP49 protein from hyperthermophilic methanogen Methanopyrus kandleri (mTIP49) as a model suitable for biochemical and biophysical analysis, and characterized its structural and catalytic properties. The rate of ATP turnover by the wild-type mTIP49 was similar to human TIP49 proteins. The deletion of the OB-fold (a structural element that is responsible for DNA binding) or the insertion of a less disruptive lik-like mutation in this domain increased the rate of ATP turnover of the mutant proteins, confirming previous experimental observations and our structural analysis of human TIP49 proteins. To rule out possible contaminations from bacterial host, we introduced an active site mutation in the Walker B motif by D206N substitution that results in the negative charge elimination within the Walker B motif. This mutation reduced rate of ATP hydrolysis to the background level observed in the absence of protein. Molecular dynamics simulations of mTIP49/ATP/Mg2+ complexes, performed in a periodic water box at the optimal for ATP hydrolysis temperature, showed DNA/TIP49a complexes can be obtained without any steric clashes with the protein loops that protrude in the central channel area. In this complex, the dsDNA helical axis is tilted by ~12° relative to the central axis of the TIP49a hexameric ring structure. The dsDNA/protein interface is formed by flexible protein loops that form intermolecular contacts with DNA. We next assessed the conformational stability of this nucleoprotein complex by molecular dynamics (MD) simulations in a periodic water box as described in (Petukhov et al., Structure, 20(8) 1321). We have found that the dsDNA fragment in the central channel of the protein ring structure was significantly stretched, with its helical pitch increased by ~23% from 34A to 42A. This was concomitant with the formation of a network of hydrogen bonds between the negatively charged DNA backbone and positively charged residues within the central channel. The resulting dsDNA structure shows tilted nucleotide bases with respect to the axis of the double helix, which are similar to that of previously reported for dsDNA overstretching in silico. In the course of MD simulations, the termini of short dsDNA fragments were curved, and found to form direct contacts with the D2 structural domain on one side of the protein ring and with the C-terminal residues on the opposite side. We also report mass-spectrometry mapping of the TIP49a residues that are involved in direct contacts with dsDNA. These MS data are in a good agreement with the results of molecular modeling. We discuss how these findings provide new insights into the question of how TIP49 ring assemblies may bind double-stranded nucleic acids.
rapid dilatation of the ATP binding pocket and loss of important polar interactions with ATP and Mg
+ in the D296N mutant compared to the wild-type hexamers. Our analysis of water dynamics within catalytic pockets of the wild-type and mutant mkTIP49 proteins leads to novel questions concerning trans-acting water-activating residues, which may serve as proton-acceptors in TIP49 oligomers, and suggests a particular mechanism of ATP hydrolysis by the TIP49 AAA+ ATPases.

**SW02.S8–22**

**Erythrocyte remodeling in acute pancreatitis**

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Acute pancreatitis is a clinical condition characterized by abdominal pain and biochemical abnormalities that develop in association with active inflammation of the acinar cells of the pancreas.

The aim of the study was to examine the erythrocyte membranes of 117 patients with acute pancreatitis. Two study groups comprising 58 patients with acute biliary pancreatitis (ABP) and 59 patients with acute non-biliary pancreatitis (ANBP) were compared. Twenty-one age and sex matched volunteers were used as control group. The various erythrocyte membrane proteins were separated by electrophoresis on Zet-Blue gels according to molecular size.

On admission, patients with ABP had abnormal protein spectrum, in which only glyceraldehyde 3-phosphate dehydrogenase and glutathione-S-transferase levels were within the physiological limits. According to current understanding of the structure of erythrocyte membrane, decreased values of a- and b-spectrin, ankyrin make the erythrocyte membrane less stable, while increased proportions of band 4.1, 4.2, 4.9, actin and tropomyosin cause loss of cell elasticity and deformability. Elevated levels of anion exchanger protein, band 4.5 and intracellular malonyldialdehyde concentration are the signs of the disordered metabolic activity of the red blood cells.

In patients with ANBP the protein spectrum is changed to a much greater extent. Most if not all the membrane proteins separable on SDS gels are damaged. The spectrin-actin network of erythrocytes is coupled to the membrane bilayer primarily by association of spectrin with ankyrin, which in turn is bound to the cytoplasmic domain of the anion exchanger. Tropomyosin is a candidate to function as a morphometric ruler defining the length of actin filaments. Defects in membrane associations result in loss of unsupported phospholipid bilayer.

Although the basic structural principals established in erythrocytes are likely to apply in other tissues, the organization, protein interactions, and functions of spectrin-based structures are considerably more specific in the red blood cells. Understanding of the molecular basis for erythrocyte membrane adaptations in acute pancreatitis represents an interdisciplinary challenge involving biochemistry, cell biology, physiology, and molecular medicine.

**SW02.S8–23**

**Asymmetric flexibility of a homodimeric enzyme as shown by molecular dynamics computations. A case study of the cold-active Vibrio alkaline phosphatase**

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Protein dynamics influence protein function and stability by modulating conformational changes. Non-covalent intramolecular interactions play a key role for such motions. The total effect will depend on the underlying networks of communicating residues within the structure. Interactions and coupled motions over the interface between subunits may further drive the catalytic cycle asymmetrically, as has been suggested for the dimeric alkaline phosphatase (AP). In this case, a conformational change might be the rate-limiting step rather than the chemical transformations. Here, we present a characterization of the dynamic properties of the cold-adapted *Vibrio splendidus* AP (VAP). Multiple all-atom explicit solvent molecular dynamics simulations were employed in conjunction with different metrics to analyze the dynamics patterns and the paths of intra- and intermolecular communication. The comparison of the dynamic patterns of the two subunits in the dimeric structure pointed out a different distribution of intramolecular interactions and correlated motions. The paths of long-range communications mediated from the catalytic residues to distal sites were also characterized, suggesting a different information flow in the two subunits. VAP displayed a low number of intersubunit interactions and coupled motions between the two halves were also few. Our results provide a structural rationale to support the half-of-site mechanism for VAP as previously proposed for some other APs. The methods may lead to characterization of asymmetric dynamics in other homodimeric enzymes.

**Reference**


**SW02.S8–24**

**Structural properties of DNA glycosylases repairing 8-oxoguanine: a molecular dynamics study**

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It is well known that DNA is highly prone to damage induced by various physical and chemical agents. Base oxidation is a prevailing damage type arising on exposure to reactive oxygen species, which leads to mutations, cell death due to stalled replication, and regulatory dysfunction, ultimately resulting in aging, carcinogenesis and many pathological processes. One of the primary pathways for dealing with such damage is base excision repair, initiated by damage-specific enzymes, DNA N-glycosylases.

The most common damaged DNA base, 8-oxoguanine (OG), is highly mutagenic because it can form a stable Hoogsteen pair with adenine. A system to prevent such mutations, known as GO-system, is possessed by both prokaryotes and eukaryotes. The GO-system comprises three enzymes, one of which, Fpg DNA glycosylase, excises OG from GO/C pairs in bacteria. Eukaryotes, including humans, possess a functional analogue of Fpg, OGG1. The X-ray structures of both these glycosylases are known.

Molecular dynamics (MD) simulation nowadays is an essential part of biochemical research. We combine a classic MD simulation with a newly developed efficient trajectory analysis tool, MDTRA, to analyze the specificity of Fpg for the base opposite to the lesion. A number of structural features have been identified making cytosine rather than adenine a preferred opposite base. Furthermore, DNA distortion by adenine opposite OG in the complex with Fpg is found to be long ranged, and even the positioning of an everted OG in the active site depends on the type of an opposite base.
In order to understand the importance of interactions within a base-binding pocket for OGG1 glycosylase, the dynamics of two models with a bulky substituent inside it (mutants C253I and C253L) was investigated. According to a known glycosylase reaction mechanism, the key parameters of catalytically important amino acids (Lys249 and Asp268) were checked. Analysis of populations of catalytically favorable conformations revealed the fact that the mutant forms should expose a reduced glycosylase activity, though not forfeiting it at all. Indeed, further pre-steady-state and steady-state kinetic experiments verified this inference.

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**SW02.S8–25**

**Dynamics of the ligand binding domain of PPAR gamma**

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 Peroxisome proliferator-activated receptor gamma (PPAR gamma) is a nuclear receptor that plays an important regulatory role in lipid metabolism, insulin sensitivity and adipogenesis. The protein harbors a large ligand binding pocket, which is a target for the development of high affinity ligands. Noteworthy among these are thiazolidinediones, a novel class of compounds that have been introduced for the treatment of type 2 diabetes, albeit with certain side effects. Over a hundred structures of the ligand binding domain of PPAR gamma are currently available from the Protein Data Bank (PDB). This includes ligand-free apo structures and structures with agonists, partial agonists and antagonists bound. In the vast majority of these structures, there is relatively very little global structural change while accommodating the ligand. A comparative study of the PPAR gamma ligand binding domain was done by performing multiple 100 ns molecular dynamics simulations of several PPAR gamma structures to elucidate the conformational dynamics of the bindingsite. The structures remained stable during the entire duration of the simulations. The apo structure exhibited a higher fluctuation of backbone heavy atoms compared to ligand bound structures. The dynamics of the residues that interact with ligands in the binding site was also studied. Several water sites were found to be conserved in the binding pocket in ligand free simulations that could be exploited for drug design.

**SW02.S8–26**

**The effect of the arthrogryposis-causing Arg91Gly mutation in beta-skeletal tropomyosin on its position on the thin filament and flexibility during the ATPase cycle**

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Previous studies have shown that the distal arthrogryposis-causing Arg91Gly mutation in β-tropomyosin (TM) alters contractile regulation. To investigate how the substitution of residue Arg91 of β-TM with a Gly residue affects the position and flexibility of TMs on the thin filaments, we labelled recombinant wild type and mutant β-TMs with 5-IAF and F-actin with FITC-phaloidin and incorporated them into the ghost muscle fibres. The orientation and mobility of the probes were studied by polarized fluormetry at different stages of the ATPase cycle. Multistep alterations in the position and flexibility of β-TM strands and actin monomers in the thin filaments during the ATPase cycle were observed. In the thin filaments the flexural rigidity of F-actin was more than twice lower than the rigidity of either of TMs. In the absence of myosin heads the mutation in TM was found to transfer tropomyosin strands towards the inner domain of actin, exposing more myosin-binding sites. Myosin heads shift the mutant TM further towards the open position, markedly changing the flexural rigidity of F-actin and TM strands and switch actin monomers on. This indicates that the amount of strongly bound cross-bridges increases at all the mimicked stages of the ATPase cycle. These structural changes in the thin filament are likely to underlie the observed rise in Ca2+-sensitivity caused by this mutation, which initiates the disease remodeling. The work was supported by the Russian Foundation for Basic Research (№ 11-04-00244a), the Programme of Presidium of RAS (theme № 7) and the Muscular Dystrophy Campaign.

**SW02.S8–27**

**Disruption of ionic interactions between NBD1 and M domain in Hsp104 chaperone unleashes toxicity to yeast cells**

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The yeast protein Hsp104 and its bacterial homologue CipB belong to the Hsp100 family and AAA+ superfamily. Genes coding these proteins are not essential at physiological conditions. They become important at heat shock conditions. Their presence determines development of thermotolerance. Hsp104 and CipB in cooperation with Hsp70 chaperone system, were shown to disaggregate and refold proteins denatured during heat stress. Hsp104 and CipB have similar structure comprising of N-terminal domain, two nucleotide binding domains (NBD1 and NBD2) and M-domain, characteristic for disaggregating chaperones from Hsp100 family.

Based on the structural analysis we proposed that the net of ionic interactions on the interface between NBD1 and M-domain is important for Hsp104. The residues D184, K358 (both NBD1), D484 (M-domain) in Hsp104. Disruption of predicted ionic interactions by introducing mutations K358E or D484K in Hsp104 caused toxic effect in yeast. Additional mutations which restore the postulated ionic interaction namely D184K/K358E and K358E/D484K or D184K/K358E/D484K resulted in lack of the toxicity. To perform more detailed analysis of growth impairment we cloned toxic mutants under control of inducible promoter. Our analysis suggests that the net of ionic interactions on the interface between NBD1 and M-domain in disaggregating chaperones is crucial for their functions. The basic, conserved residue (K358 in Hsp104) is the most important in forming the discussed net of interactions.

**SW02.S8–28**

**Molecular dynamics study of A-domain of Protein Kinase A I -alpha?**

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Though cAMP-binding A-domain of Protein Kinase A I is thoroughly studied, there is no model accounting for its transition from H- (cAMP-free) to B- (cAMP-binding) conformation.
Moreover, contradictory data exist regarding the cAMP-binding residue at position 209. While some authors presume that R209 is invariant, others suggest that this arginine can be replaced by several residues (such as I or T, but not E and K) with little loss of protein kinase function. We undertook molecular dynamics (MD) analysis of these two options. At first, MD simulations were performed on truncated (150–225 a.a., without C-helix and N3A-motif) A-domain in the presence or absence of cAMP with subsequent data processing by Cluster Analysis. The results proved that transition to B-conformation doesn’t need cAMP (if domain isn’t restrained to H-conformation by other proteins) and showed that correct conformation of b2b3-loop is necessary to reach B-conformation. The second part of the study was performed with the same methods on the same truncated systems but carrying point mutation at position 209 (I, G, E or K instead of R). According to our results, only R209I and R209E mutants could maintain correct b2b3-loop conformation and only R209I and R209K mutants could keep cAMP in the binding site. However, if external forces were applied to b2b3-loop, all the mutants except for R209K underwent transition to B-conformation. Lysine at position 209 not only distorted b2b3-loop twist, but also sterically interfered with A202 movement, which is crucial for domain transition. If both b2b3-loop and K209 were fixed by external forces, transition to B-conformation was observed. The third part of the study deals with MD and activated MD simulations of non-truncated A-domain (118–242 a.a.) with subsequent data processing by Principle Component Analysis. We demonstrated that main stages of A-domain transition from H- to B-conformation involve Phosphate Binding Cassette conformational changes, C-helix rotation, formation of C-helix turn within C-helix, and finally substitution of this turn by a kink. R209K mutant goes through the same stages, provided that K209 and b2b3-loop are fixed by external forces as shown for truncated systems.

**SW02.S8–29**

Large-scale mobility of RecA protein filaments in solution by molecular dynamics simulation and neutron spin echo

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RecA [1,2] protein plays key role in homology recombination reaction in bacterial cell. It works by forming right-handed filament structure on ssDNA [3]. RecA searches homology region between two DNA molecules: ssDNA inside RecA filament and dsDNA outside it, and then it does strand exchange reaction between this two homology regions.

Based on the data available in PDB [2,4] we built a full atomic model of the protein filaments formed by RecA from E. coli and D. radiodurans, consisting of 12 monomers. To investigate large scale conformational motions of these two proteins a molecular dynamics simulations using GROMACS [5] molecular dynamics package and the corresponding neutron spin-echo experiments on J-NSE (Muenchen, Germany) and IN15 (Grenoble, France) spectrometers were performed. The results of the simulation reveal the role of domain mobility of RecA monomer in the different filament motion modes and were generally in agreement with the results of neutron scattering experiments.

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**References**


**SW02.S8–30**

Mechanisms of human fibrinogen adsorption on colloidal particles determined by electrokinetic and AFM measurements

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Adsorption of plasma proteins is the key event following blood biomaterial contact. These protein films influence biological events like platelet adhesion and thrombogenesis on biomaterial surfaces. Fibrinogen is the third most abundant protein in plasma and play an important role in the development of surface induced thrombosis. Therefore, the knowledge of fibrinogen adsorption mechanisms at solid/electrolyte interfaces is of major practical significance. This provoked much research aimed at determining its properties in bulk and interactions with various surface.

The adsorption of human fibrinogen on polystyrene latex particles was studied using the microelectrophoretic method. Measurements were carried out for pH 3.5 and an ionic strength range of 10−3–0.15 M NaCl. The electrophoretic mobility of latex was determined as a function of the amount of adsorbed fibrinogen. The maximum coverage of fibrinogen on latex particles was determined using the depletion method. The residual protein concentration after making contact with latex particles was determined by electrokinetic measurements and AFM imaging where the surface coverage of fibrinogen on mica was quantitatively determined. A monotonic increase in the zeta potential of the latex was observed for prolonged time periods, suggesting the irreversibility of fibrinogen adsorption. Therefore, the experimental data agree with theoretical simulations performed by assuming a 3D unoriented adsorption of fibrinogen. The stability of fibrinogen monolayers on latex was also determined in ionic strength cycling experiments. It was revealed that cyclic variations in NaCl concentration between 10−3 and 0.15 M induced no changes in the latex electrophoretic mobility, suggesting that there were no irreversible molecule orientation changes in the monolayers. On the basis of these experimental data, a robust procedure of preparing fibrinogen monolayers on latex particles of well-controlled orientation and coverage can be envisaged, which is significant for various immunological assays.
SW02.S8–31
Human AP endonuclease 1 active site plasticity: MD simulation of WT and mutant enzyme-substrate complexes
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Understanding the molecular mechanisms of protein-nucleic acids interactions is a primary goal of modern structural biology. Molecular basis of its specificity lies in conformational properties of a protein, its target DNA site, and the changes that ensue as a consequence of their interaction.

Apurinic/apyrimidinic (AP) sites are abundant DNA lesions arising from exposure to UV light, ionizing radiation, alkylating agents, and oxygen radicals. In human cells, AP endonuclease 1 (APE1) recognizes this mutagenic lesion and initiates its repair via a specific incision of the phosphodiester backbone 5′ to the AP site.

The structure and mechanism of APE1 has been studied extensively, and there are nine crystal structures available in the PDB databank.

In this study, we analyzed the substrate specificity and the catalytic mechanism of APE1 acting on various DNA substrates using molecular dynamics simulation. We have studied both the complex of WT APE1 with AP-site containing DNA and the complexes of various mutant forms of the enzyme: K98A, N212D and N212A. DNA duplexes, on the other hand, contain 2-aminopurine residue next to the AP-site. The dynamic changes in these complexes have been studied previously using stopped-flow technique (Kanazhevskaya L. Yu. et al. 2012, Timofeyeva N.A. et al. 2011).

Using detailed molecular dynamics simulations of the WT and mutant enzyme-substrate complexes, we have attributed structural distortions of AP-DNA to realization of specific binding, effective locking, and incision of the damaged DNA. The role of enzyme and DNA conformational flexibilities has been established in connection with the accessibility of the active site, the binding of substrates and ligands, and release of products, stabilization and trapping of intermediates, orientation of the substrate into the binding cleft, adjustment of the reaction environment. These findings allowed us to consider APE1-DNA complexes as networks of continuous motions, which reflect local flexibility and ability for global structural plasticity.

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SW02.S8–32
Expression and immunohistochemical distribution of mitogen-activated protein kinases in normal and pathological placental tissues
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Mitogen-activated protein kinases (MAPKs) play the main role in the signal transduction of many cellular events, such as embryogenesis, cell proliferation, differentiation and cell death. They are also critical for placental development and some placental defects such as insufficient oxygen and nutrient transfer occur in their knock out forms. Moreover, little is known about the expression and activation of classical MAPKs in normal and pathological human placental tissues. Therefore, in this study, we examined the expression of both phosphorylated and total forms of classical MAPKs, ERK1/2, JNK and p38, in normal, diabetic and preeclamptic human term placental tissues using Western blotting and immunohistochemistry. The immunohistochemical distribution patterns of phosphorylated and total forms of ERK1/2 and JNK were similar and localized in syncytiotrophoblast, endothelial cells, villous connective tissue cells, amniotic epithelial cells, extravillous cytotrophoblasts and decidual cells. Moreover, total ERK1/2 was stained also in villous cytotrophoblasts. Phosphorylated and total forms of p38 were mainly found in villous cytotrophoblasts and extravillous cytotrophoblasts. Immunohistochemical results showed that expression of all MAPKs were gradually decreased through normal, preeclamptic and diabetic placental tissues. In addition, Western blot analysis showed that stronger bands were obtained in control placental tissues and band intensity decreased in various degrees in both preeclamptic and diabetic placental tissue samples for all MAPK antibodies. These findings suggest that MAPK pathway is necessary for normal placental tissue and decreased expression of MAPKs in diabetic and preeclamptic placental tissues might contribute to their placental defects and pathogenesis.

SW02.S8–33
The kinetic study of human apurinic/apyrimidinic endonuclease 1 in nucleotide incision repair pathway
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The major human apurinic/apyrimidinic endonuclease 1 (APE1) is a key enzyme in the base excision repair (BER) and nucleotide incision repair (NIR) pathways. BER is initiated by DNA glycosylases, excising the damaged and/or mispaired bases to produce apurinic/apyrimidinic sites (AP sites). AP sites are generated also through the spontaneous loss of bases. During BER pathway DNA in human cells is hydrolytically nicked 5′ to the AP site by APE1. Repair of certain base lesions can be initiated directly by the AP endonucleases alone in NIR, by-passing the DNA glycosylase step. During this process, an AP endonuclease introduces a nick 5′ to the damaged deoxynucleotide, generating a 3′-hydroxyl terminus and a 5′-phosphate terminus. Using a stopped-flow fluorescence method we analyzed the conformational dynamics and kinetic mechanism of wild-type APE1 and its mutants APE1K98A and NA61APE1. DNA substrates used in this study contained AP site, tetrahydrofuran, 5,6-dihydrouridine (DHU) or N2′-deoxyadenosine. Our data suggest that APE1 can pre-exist in two conformations and that the conformational selection and induced fit occur during the enzyme action. The enzyme release from the complex with the nicked DNA product limits the overall NIR process and determines its rate in the steady-state conditions. The comparison of the kinetic constants of mutants to those of wtAPE1 provides us with a view of the roles of lysine-98 and of REF1 domain. We have shown that during both BER and NIR pathways Lys98 is important in the 5′-phosphodiester bond hydrolysis of DNA substrate. The REF1 is required for the
SW02 Biocatalytic Mechanisms and Protein Dynamics

**SW02.S8–35**

**Analysis of molecular dynamic the second catalytic cysteine half-domain (SCCH) from ubiquitin-activating enzyme E1 based on 15N relaxation data in solution**

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Process degradation of protein require concerted action of three enzymes responsible for activation (E1), conjugation (E2) and ligation (E3). As well known, the mechanism of activating E1 enzyme involve adenylation of C-terminal glycine in ubiquitin followed by activation of catalytically-active cysteine in E1 enzyme. The catalytic domain of ubiquitin E1 consist two stable half-domains – first (FCCH, 2V31) and second (SCCH, 1Z7L) catalytic cysteine half-domains [1] (pdb 2V31, 1Z7L). In spite of availability 3D structures of FCCH, SCCH, as well as whole east E1 enzyme (3CMM) [2] there are some critical points described of ubiquitin-activation process still have to be clarified.

In presented work, we reported analysis of molecular dynamic processes in recombinant SCCH (276 residues long) fragment as well as it SCCH (C706S, C711S, C719S, C748S) mutant specifically synthesized for covalently disulfide bonding with ubiquitin [3]. For this purpose, 15N relaxation data (R1, R2, and (1H,15N) NOE) were acquired on 18.8 T with Agilent VNMR 800 NMR spectrometer at 280 K for both NMR samples. Analysis experimental data were performed with ModelFree approach and Spectral Density Mapping formalism based on previously achieved assignments [4]. The extracted data for 203 assigned signals provide to overall correlation time is 20.5 ns. The initial analysis exhibits possible structural alterations in L733 – G744 mobile loop and two z-helices (I641 – F656 and N720 – N728) which are rearranged under ubiquitin-activating process. More detail analysis will be presented on poster.

**Acknowledgements:** This work is financially supported by the grant from Polish Ministry of Higher Education N N301 318539 and European FP7 projects EastNMR (contract no. 228461) and BioNMR (contract no. 261863) inside of transnational access programs. IZ has partial financial support from EN-FIST Center of Excellence (www.en-fist.si). EL is recipient of European Erasmus exchange program and FEBS Collaborative grant.

**References**


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**SW02.S8–34**

**Thermal transitions of bacterial bioluminescence enzymes in viscous media by means of their intrinsic fluorescence**

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Viscous and gelatinous media can be considered as the simplest models of intracellular environment for the enzymes. To reveal the peculiarity of enzymes functioning in vivo the mechanisms of bacterial bioluminescent reaction in viscous media have being investigated. Earlier it was found that by addition of glycerol and sucrose into medium the intensity of bacterial bioluminescent reaction in viscous media have being investigated.

The work was supported by grants from the RAS Program ‘Molecular & Cell Biology’ [6,11], RFBR [13-04-00013] and RMES [SS-64.2012.4, 8092, 8473, 11.G34.31.0045] to N.A.T., V.V.K. and O.S.F, as well as grants [ANR Blanc 2010 Project ANR- 09-GENO-000, PICS N5479, RB 2013] to M.K.S. and [#2012 00029161] to A.A.I.

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**SW02.S8–35**

**Analysis of molecular dynamic the second catalytic cysteine half-domain (SCCH) from ubiquitin-activating enzyme E1 based on 15N relaxation data in solution**

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Process degradation of protein require concerted action of three enzymes responsible for activation (E1), conjugation (E2) and ligation (E3). As well known, the mechanism of activating E1 enzyme involve adenylation of C-terminal glycine in ubiquitin followed by activation of catalytically-active cysteine in E1 enzyme. The catalytic domain of ubiquitin E1 consist two stable half-domains – first (FCCH, 2V31) and second (SCCH, 1Z7L) catalytic cysteine half-domains [1] (pdb 2V31, 1Z7L). In spite of availability 3D structures of FCCH, SCCH, as well as whole east E1 enzyme (3CMM) [2] there are some critical points described of ubiquitin-activation process still have to be clarified.

In presented work, we reported analysis of molecular dynamic processes in recombinant SCCH (276 residues long) fragment as well as it SCCH (C706S, C711S, C719S, C748S) mutant specifically synthesized for covalently disulfide bonding with ubiquitin [3]. For this purpose, 15N relaxation data (R1, R2, and (1H,15N) NOE) were acquired on 18.8 T with Agilent VNMR 800 NMR spectrometer at 280 K for both NMR samples. Analysis experimental data were performed with ModelFree approach and Spectral Density Mapping formalism based on previously achieved assignments [4]. The extracted data for 203 assigned signals provide to overall correlation time is 20.5 ns. The initial analysis exhibits possible structural alterations in L733 – G744 mobile loop and two z-helices (I641 – F656 and N720 – N728) which are rearranged under ubiquitin-activating process. More detail analysis will be presented on poster.

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**References**

**SW02.S8–36**

**Why some cells are radioresistant? The DBp53 interactions with double-strand DNA sequences in promoter regions of genes related to the cell cycle and apoptosis**

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The p53 protein is a guardian of genome and plays decisive roles about the fate of a cell after its DNA gets damaged. Because of mutations the DNA-binding domain of p53 (DBp53) is inactivated in about 50% of human cancer cells. The DBp53 specifically interacts with double-strand DNA molecules within promoters regions of genes related to the cell cycle and apoptosis. These DNA targets consists of at least two decameric motifs of a general form **RRRCWWGYYY** \((R = A,G; W = A,T; Y = C,T)\), separated by 0–13 base pairs [1]. For the studies, the promoter regions of the genes of three proteins were selected, namely p21 (involved in G0 phase arrest and cell cycle repair), gadd45 (G2-M phase arrest, the cells are the most sensitive towards irradiation) and pig3 (initiates generation of ROS to push the cell on G1/S transition pattern, the average number of water molecules bound to a DNA duplex (and released upon melting) was significant change of a CD spectrum of the DNA component was observed for interactions GADD45/DBp53. This phenomenon probably depends more on a secondary structure of GADD45 duplex (perhaps the shape of the major groove) than on the flexibility of the duplex. As the flexibility is correlated with the hydration pattern, the average number of water molecules bound to a single base-pair in the duplex (and released upon melting) was assessed using a UV method and an osmotic stress method [2].

Upon gamma irradiation glutathione is supposed to get oxidized to protect the cell from oxidation-related damage [3]. By mass spectrometry, increased formation of disulfide bonds between added oxidized glutathione (GSSG) and sulfhydryl groups in DBp53 was observed (from 2 to 9 glutathionylated cysteins).

**References**


**SW02.S8–37**

**Prediction of status residue to be protected or unprotected from hydrogen exchange in a protein chain**


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To clarify the relationship between structural elements and polypeptide chain mobility, a set of statistical analyses of structures is necessary. Since at present proteins with determined spatial structures are much less numerous than those with amino acid sequence known, it is very important to be able to predict the extent of proton protection from hydrogen-deuterium exchange basing solely on the protein primary structure. Here we present a novel web server aimed to predict the degree of amino acid residue protection against hydrogen-deuterium (HD) exchange solely from the primary structure of the protein chain under study. On the basis of the amino acid sequence of a globular protein, the presented server offers the following three possibilities (predictors) for user’s choice. First, prediction of the number of contacts occurring in this protein, which is shown to be helpful in estimating the number of protons protected against HD exchange (sensitivity 0.71). Second, probability of H-bonding in this protein, which is useful for finding the number of unprotected protons (specificity 0.71). The last is the use of an artificial predictor. Also, we report on mass-spectrometry analysis of HD exchange that has been first applied to free amino acids. Its results showed a good agreement with theoretical data (number of protons) for ten globular proteins (correlation coefficient 0.73). We pioneered in compiling two data-sets of experimental HD exchange data for 35 proteins. The H-Protection server is available for users at http://bioinfo.protres.ru/opp/.

This study was supported by the Russian Foundation for Basic Research and by the Russian Academy of Sciences programs ‘Molecular and Cell Biology’ and ‘Fundamental Sciences to Medicine’.

**SW02.S8–38**

**An asymmetric transition between symmetric states: the Glucosamine 6-phosphate Deaminase allostery**

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Allosteric systems are characteristic of proteins with one or more equilibrium states. Such an enzyme experiences a modification of its activity when a cooperative substrate binds to a state or another, thus, establishing a change in population equilibrium. X-ray diffraction and NMR experiments already demonstrated this dynamic equilibrium, and simulation methods, as molecular dynamics and normal mode analysis, generally provide a more complete description.

The allosteric enzyme Glucosamine-6-Phosphate Deaminase (GlcN6PD) appeared to be a good model to better understand the equilibrium dynamics as essential to the protein function, given its reversibility of the catalysis, its relative small size and rapid-equilibrium kinetic mechanism. It also has the structure elucidated for both its conformers. A computational approach would now give better perspective on how the conformational changes occur. A set of results of this latter kind were obtained: (i) a 100 ns dynamic starting at the hexameric T conformer, explicitly solvated, building a NVT ensemble using NAMD program and CHARMM force field; (ii) and normal mode analysis of the T conformer; (iii) a principal components analysis making use of the calculated dynamic; (iv) and normal mode analysis of the T conformer structure.

As the allosteric transition is described, in theory, as only tertiary and quaternary structural changes, analysis taken here aimed for global movements, or the displacements of some characteristic regions that act like rigid body subunits. Hence, inertia tensor analysis were developed, since it should act as a filter for the high frequency and functionally uninteresting motions. These analysis indeed showed the GlcN6PD passing through conformational changes that correspond to the allosteric transition. Within 100 ns of a molecular dynamics and in the normal mode analysis, structural analysis showed that all its six protonomers reached the R state, being capable, at this point, of holding a
ligand in the allosteric site (as simulated in a 20 ns dynamics). Although, different from the expected by Monod’s theory, the six protoners did not make the transition all together in a symmetrical way. Nevertheless, energy analysis revealed that the energy required to these structural changes to occur is well provided by the thermal bath, being less than kT.

SW02.S8–39
FTIR spectroscopy applied to study dynamics and flexibility of human aromatase
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Human aromatase (CYP19A1) is the cytochrome P450 involved in the steroidogenesis by converting androgens into estrogens. The crystal structure of the enzyme in complex with the substrate androstenedione and the steroidal inhibitor exemestane, showed a very compact conformation of the protein that however is able to accommodate even molecules structurally very different from the steroids. Crystal structures of the protein in the absence of ligands or in the presence of non-steroidal inhibitors such as anastrozole, a drug currently used for the treatment of breast cancer in post-menopausal women, are not available until now.

In this work, FTIR was applied to follow H/D exchange and therefore to study the flexibility of the ligand-free enzyme in comparison to the forms complexed with the substrate (androstenedione) and the non-steroidal inhibitor anastrozole. In particular, the signal in the amide I region was used to specifically follow the H/D exchange of the different elements of secondary structure and measure exchange rate constants.

The results show that the presence of the substrate androstenedione and the inhibitor anastrozole has the effect to significantly lower the H/D exchange rates \( k_1 \) and \( k_2 \) of the \( \alpha \)-helices of the enzyme. Furthermore, the presence of the inhibitor was also to lower the exchange rate \( k_1 \) for \( \beta \)-sheets from 0.221 ± 0.055 to 0.119 ± 0.017/min, respectively.

Time resolved fluorescence experiments show a shift in \( \tau_s \), detected at 5.1 ns and assigned to Trp224 by site-directed mutagenesis, toward longer lifetimes when the substrate and the inhibitor are present. These data show that the dynamics of Trp224, located in helix F and part of the ligand access channel, is altered upon substrate and inhibitor binding.

The results are consistent with an increase in access channel flexibility in aromatase when a ligand is not present, that is necessary to accommodate the steroidal substrates and also non-steroidal ligands such as anastrozole.

SW02.S8–40
Antibody opsonization-dependent internalization and trafficking of ABCG2 in cancer cell lines
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Human ABCG2 known also as breast cancer resistance protein (BCRP) is a plasma membrane half-transporter belonging to ABC superfamily. As an efflux pump it is functional predominantly in barrier tissues, progenitor cells and in the liver, playing important role in protection against xenobiotics. It is also claimed to be one of the factors responsible for clinical multidrug resistance in cancer.

In our research, we have found a new trafficking mechanism for the human ABCG2 protein, consisting of endocytosis dependent on antibody opsonization. Upon incubation with 5D3 antibody that binds to an extracellular epitope, the majority of BCRP present in plasma membrane is translocated to an internal vesicular compartment and subsequently trafficked within intracellular organelles. We observe this in various cancer cell lines of different origins expressing varying amounts of endogenous BCRP, as well as in cells that were stably or transiently expressing heterologous ABCG2. Experiments with known inhibitors of the endocytosis prove that this process is cholesterol- and dynamin-dependent, but probably clathrin- and caveolin-independent. As such, we cannot yet clearly classify it to any of the canonical endocytic pathways. However, within 24 h after endocytosis, internalized protein-antibody complex seems to be degraded only to a small extent, with some of it moved back to the plasma membrane and a major part localized in a yet unknown, non-lysosomal intracellular compartment, where it is retained.

The ability of ABCG2 to undergo endocytosis without subsequent degradation is interesting as a potential regulatory mechanism for its physiological function on small timescales. Here we show the first evidence of complex reversible endocytosis similar to that demonstrated for other apical ABC transporters (e.g. ABCC2). However, the detailed molecular mechanism of this endocytosis and the eventual fate of the intracellularly sequestered protein are divergent from canonical pathways described for other membrane transporters and should be studied in more detail.

SW02.W9 Enzymes Reacting with Organophosphorus Agents (II-W9)

SW02.W9–1
Reaction of tyrosinyl, histidinyl and lysinyl residues with OP
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Acute toxicity from OP exposure is due to inhibition of acetylcholinesterase activity. However, illness from OP exposure is not always associated with acetylcholinesterase inhibition; for example tricresyl phosphate exposure is associated with inhibition of neurotoxic esterase. Furthermore, toxic signs differ depending on the identity of the OP. These observations have led to the hypothesis that proteins in addition to acetylcholinesterase are modified by OP. In support of this hypothesis, a variety of proteins that are reactive toward OP have been identified including butyrylcholinesterase, albumin, acetylpeptide hydrolase, and tubulin. Blood samples from human subjects who attempted suicide by ingesting chlorpyrifos or dichlorvos were analyzed by mass spectrometry for the presence of OP labeled proteins. The subjects had OP adducts on tyrosine 411 of albumin and on serine 198 of butyrylcholinesterase. These results proved that albumin is sufficiently reactive with OP to yield adducts in living humans. Mice treated with doses of chlorpyrifos too low to inhibit acetylcholinesterase, had OP-modified tubulin in brain. Rats treated with paraoxon had OP modified albumin in blood. In vitro studies with purified proteins revealed that OP made stable covalent bonds on serine, tyrosine, histidine and lysine. Methods have been developed to enrich plasma samples for OP modified peptides, including binding of aged OP-butyryl-
Abstracts

**SW02.W9–2**

Structural dynamics of acetylcholinesterase as studied by kinetic crystallography


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The delicate balance between structure and dynamics allows proteins to be biologically active. We aim at understanding the structural dynamics involved in the molecular traffic and inhibition of acetylcholinesterase (AChE) by applying X-ray crystallography and complementary biophysical methods and molecular dynamics simulations. Whereas X-ray crystallography in general provides static pictures of a protein, kinetic crystallography unravels its structural dynamics. In particular, temperature-controlled kinetic crystallography offers the possibility to generate, trap and visualize enzymatic reaction intermediates [1]. By recruiting X-ray induced modifications of AChE in temperature-controlled kinetic crystallography experiments, we were able to provide experimental evidence for the existence of a ‘backdoor’ involving Trp84 in Torpedo californica (To)AChE [2]. More recent crystallographic data point to the interface between Tyr442 and Trp84 as the key element in backdoor opening [3]. Beyond academic interest, studying the structural dynamics of AChE benefits the rational design of inhibitors and reactivators, including anti-Alzheimer drugs and antidotes against organophosphate poisoning, respectively.

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**References**


**SW02.W9–3**

QM/MM of ChE-catalyzed reactions with special attention to OP inhibition

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We performed molecular dynamics and QM/MM studies of the G117H mutant of BuChE hydrolyzes OPs at slow rates. Understanding of the hydrolytic mechanism of bound OP should help to improve the OPase activity of ChE-based catalytic bioscavengers. The crystallographic structure of the G117H mutant of BuChE conjugated to echotoxiphe was solved recently [1].

We performed molecular dynamics and QM/MM studies of the G117H BuChE mutant conjugated to echotoxiphe in order to consider different hydrolysis mechanisms. The possible changes in His117 orientation, depending on protonation state, and its influence on average enzyme dynamics were considered. The hydrolysis reaction energy profiles for different possible hydrolysis mechanisms were calculated and compared with respective profiles for the aging process (dealkylation of the conjugated OP). His117 protonation lowers reaction energy barriers and stabilizes reaction products for both the hydrolysis and aging processes compared to reactions in the wild-type BuChE inhibited by echothiophate.

An OP of particular interest is cresyl saligenin phosphate (CBDP). It is considered as a potential toxicant in aerotoxic syndrome. Molecular modeling methods such as molecular docking, molecular dynamics, quantum chemistry, QM/MM and QM/MD help to address these issues. We explored the interaction of CDP with BuChE, the enzyme reactivity and possible causes for the observed biphasic kinetics of inhibition.

**References**


**SW02.W9–4**

Theoretical kinetic aspects of enzymes reacting with OPs

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Serin esterases and peptide enzymatic activity are covalently inhibited by organophosphorus compounds (OPs) and other acylating chemicals as sulphonyl fluorides (i.e. PMSF) or carbamates. Cholinesterases and neuropathy target esterase (NTE) are well known examples as well as the neurotoxicological consequence their covalent inhibition by organophosphorylation, sulphonylation or carbamoylation. The biological function of acetylcholinesterase are well known and related directly with their catalytic esterase properties but not well established for NTE. A role of
NTE protein in cell differentiation and embryonic development is under study. In mammals, avian and other species, other carboxylesterases with unknown toxicological and biological function are interacting with OPs. Kinetic models have been developed and applied for analyzing the time progressive inhibition in complex systems, containing several esterase sensitive components and considering the sensitivity (inhibition rate constant) and spontaneous reactivation (dephosphorylation). In some cases (i.e.: PMSF) the inhibitor is unstable changing the concentration along the time of testing and this should be considered in the mathematical kinetic model. Other factors to be considered in the kinetic model are: “ongoing” inhibition during the time of measuring residual activity, interactions between inhibitor in studies with two inhibitors, aging reaction of inhibited enzyme. Example are shown analyzing the esterase component on membrane and/or soluble esterases in peripheral nerve, brain and serum of chicken, the animal model for testing the OP delayed neuropathy and other neurotoxicological studies. In each tissue, several main esterase components with different properties for inhibition, reactivation have been discriminated and evaluated with the model inhibitor, paraoxon, mipafox, and PMSF, and S9B. These kinetic studies allow identifying which are the esterases that need to be molecularly identified to understand the toxicity and for developing methodology for clinical diagnostic in humans and in experimental animal studies.

**SW02.W9–5**

**Model equations of inhibition of esterases by non-stable compounds: PMSF as a model**

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PMSF is a protease and esterase inhibitor causing protection or potentiation of the organophosphorus (OP) delayed neuropathy and it is instable in water solution. A kinetic model equation was deduced assuming a multienzymatic system with three different molecular phenomena occurring simultaneously: (i) inhibition; (ii) spontaneous chemical hydrolysis of the inhibitor; (iii) ongoing inhibition (inhibition during the substrate reaction). A three-dimensional fit of the model was applied for analysing the experimental data. This model was applied to soluble fraction of brain and peripheral nerve from chicken; the animal model for testing OP delayed neuropathy. In peripheral nerve the best fitting model is compatible with a resistant component (17%) and two sensitive enzymatic entities (42% and 41%). The corresponding second order rate constants of inhibition (ki = 95.8 x 103 and 7.0 x 103 M⁻¹ min⁻¹, respectively) and the chemical hydrolysis constant of PMSF (kh = 0.0631 min⁻¹) were simultaneously estimated. In soluble brain fraction the best fitting model is compatible with a resistant component (14%) and two sensitive enzymatic entities (44% and 41%). The corresponding second order rate constants of inhibition (ki = 0.0076 and 0.0014 nM⁻¹ x min⁻¹, respectively) and the chemical hydrolysis constant of PMSF (kh = 0.28 min⁻¹) were simultaneously estimated. The consistency of results in fixed time and progressive inhibition experiments was considered an internal validation of the methodology. In addition changes on the sensitivity to others inhibitors has been detected when PMSF previously interacts with the esterases of brain fractions. The data suggest that these effects might be due to an irreversible interaction of PMSF at sites other than the substrate catalytic center.

**SW02.W9–6**

**NTE and neuropathies induced by OP compounds**

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Neuropathy target esterase (NTE) was discovered through a search for the molecular initiation site of the axonal degeneration brought about by certain organophosphorus compounds (OPCs). Structure-activity relationships of NTE inhibitors led to the view that the biochemical lesion of OPC-induced delayed neuropathy (OPIDN) consisted of NTE-OPC conjugates that had undergone aging. Indeed, nonaging NTE inhibitors failed to produce disease but protected against neuropathy from subsequently administered aging inhibitors. Thus, loss of NTE activity was not the culprit; rather, formation of an abnormal protein was the agent of the disorder. For the ageable OPCs, the relative potency of a compound to react with NTE versus AChE in vitro predicts its capability to produce OPIDN, and blood NTE inhibition can be used as a biomarker of exposure to neuropathic OPCs. Whereas conventional knockout of the NTE gene is embryonic lethal, conditional knockout of central nervous system NTE produces neurodegeneration, suggesting that the absence of NTE rather than its presence in some altered form causes disease. NTE is the 6th member of a 9-protein family called patatin-like phospholipase domain-containing proteins, PNPLA1-9. Mutations in the catalytic domain of NTE (PNPLA6) are associated with a slowly developing disease akin to OPIDN and hereditary spastic paraplegia called NTE-related motor neuron disorder (NTE-MND). Furthermore, the NTE protein from affected individuals has altered enzymological characteristics. Moreover, closely related PNPLA7 is regulated by insulin and glucose. These seemingly disparate findings are not necessarily mutually exclusive, but recent genetic findings need to be reconciled with the historical body of toxicological data indicating that inhibition and aging of NTE are both necessary to produce OPIDN. Elucidating the roles of NTE in genetically and chemically induced neuropathies is expected to enhance our understanding of the physiological and pathogenic roles of the PNPLA family of proteins in neurological health and disease, including a potential role for NTE in diabetic neuropathy.

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**SW02.W9–7**

**X-ray structures of phosphorylated cholinesterases**

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Organophosphorus nerve agents (OPs) exert their acute toxicity by phosphorylating the catalytic serine of acetylcholinesterase (AChE), resulting in the disruption of cholinergic transmission. Butyrylcholinesterase (BChE) is a homologous enzyme, abundant in tissues, and an efficient OPs scavenger. Administration of BChE is actually considered for prophylaxis and treatment of OPs poisoning. AChE exerts a strong enantiomerelectivity for chiral OPs while BChE is less selective. Phosphorylated cholinesterases can be reacti-
vated by oximes to some extent. However, the inhibited enzyme undergoes a first order reaction, termed ‘aging’, that progressively prevents reactivation. These features can be understood at the molecular level in the light of the X-ray structures of phosphorylated and aged cholinesterases that were solved during the last 15 years.

The enantioselectivity of AChE results from the shape of the deep active site gorge at the bottom of which the catalytic triad is located. Two sub-sites of the gorge, the acyl-binding and choline-binding pockets named according to their respective role in acetylcholine binding, differ by their dimension. The resulting chirality of the active site of AChE exerts a strong enantioselectivity on chiral OPs like nerve agents. Yet, enlargement of the acetyl-binding pocket of AChE has been observed in a few cases. The difference in pockets size is less pronounced for BChE so that enantioselectivity for chiral OPs is weaker and specificity is broader.

X-ray structures of pro-aged and aged cholinesterases show that the aging reaction generally corresponds to the O-dealkylation of one alkoxy substituent of the phosphorus atom located in the choline-binding pocket. This mechanism involves carbocation stabilization, and leads to the formation of a locking salt bridge with the catalytic histidine.

Finally, the X-ray structures of AChE in complex with bispyridinium-oximes show one pyridinium bound to the peripheral aromatic site at the entrance of the gorge and the oxime-bearing pyridinium entering the gorge. Yet the oxime orientation is not favorable to reactivation and hints that structure-based optimization of oxime reactivators is possible.

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**SW02.W9-9**

**Evolved paraoxonases against nerve agents**

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The high toxicity of organophosphorus compounds arises from the irreversible inhibition of acetylcholinesterase (AChE), an essential enzyme in cholinergic neurotransmission. Poisonings which lead to life-threatening toxic manifestations call for immediate treatment, which usually consists of a combined administration of anticholinergic drugs and an oxime as the reactivator of AChE. A new approach to reduce the in vivo toxicity of organophosphorus compounds includes the use of bioscavengers – enzymes that could react with the nerve agent before it inhibits acetylcholinesterase. Butyrylcholinesterase (BChE), naturally present in plasma, the liver, the small intestine, smooth muscles, heart, adipose tissue, and the brain, is considered an endogenous bioscavenger of anticholinesterase compounds. Due to the limited concentration of BChE in the organism, a stoichiometric reduction of nerve agents is not sufficient. Others have shown that injections of milligram quantities of purified human BChE can be efficient for stoichiometric scavenging, but are prohibitively expensive for treatment of wider populations. In addition, this stoichiometric approach has limitations mostly due to the inability of currently applied reactivators to reactivate tissue AChE efficiently, particularly when it is repeatedly phosphorylated by an excess of tabun or soman remaining in circulation upon exposure. Our recent studies have shown that AChE mutagenesis enables oximes to substantially accelerate the reactivation of the soman-enzyme conjugate which resists aging. We created a human AChE mutant, Y337A/F338A, to combine the increased accessibility of conjugated phosphorus to oximes provided by the Y337A mutation with the aging resistance of the F338A mutation. We also designed a library of new oximes where we identified efficient reactivators of the Y337A human AChE inhibited by tabun that accelerate the reactivation rate 10-times in comparison to the AChE wt. Our overall findings indicate that distinct human AChE mutants have a catalytic bioscavenging potential when combined with oximes, which has been proven effective in soman or tabun in vitro exposure.

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**References**


SW02.W9–10
The ‘thio effect’ - a kinetic barrier in OPH-catalyzed hydrolysis of P-S-R containing OPs

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Phosphoro- and phosphonothiolates [(R)(R')P(O)-X; X = S-alkyl or S-aryl], are turned over by wt OP hydrolysing (OPH) enzymes less effectively than their oxo analogs. For example, paraoxon is hydrolyzed 36-fold faster by wt bacterial phosphotriesterase (PTE) than its S homolog, parathiol, despite the >600-fold greater acidity in favor of parathiol’s p-nitrothiophenol leaving group (pK_a = 4.4). Improvement of the catalytic hydrolysis of phosphoro- and phosphonothiolates thus needs to take into consideration the P-S cleavage barrier, defined here as the ‘thio retardation effect’ (TRE). To detect trends that might reduce the TRE, a protocol was developed for its quantitative assessment, based on comparison of the enzymatic hydrolysis of a pair of diethyl phosphates and a pair of methylphosphonates bearing either a thio- or an oxo-based leaving group, X, possessing similar pK_a values. This set of OPs cancels out differences in the electronic characteristics of the leaving groups. The ratios of the k_cat/ K_m values for action on the O and S homologs were taken as a measure of the TRE. In the case of PTE a significant improvement in catalysis of the thiolo substrates was observed along the enhanced evolution process, but the O/S ratio remained similar to that of the wt enzyme (2000–3000). In contrast, in some of the mammalian paraoxonase (PON1) libraries a clear trend was noted that resulted in reduction of the O/S from ~2000 to ~200. It is conjectured that the TRE arises due to the larger dimensions of PON1 and PTE variants with improved activity on these substrates.

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SW02.W9–11
Chemical polysialylation of human butyrylcholinesterase. Towards the delivery of a long-acting bioscavenger for nerve agents in vivo

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The new generation of DNA-encoded bioscavengers were suggested recently for side effects-free pre- and post-treatment of poisoning by organophosphorous compounds (OPC). We report here strategy to generate a recombinant bioscavenger powered by chemical polysialylation, which is extremely stable in the bloodstream. We succeeded in developing of CHO-based expression system of recombinant human butyrylcholinesterase (rhBChE), delivering a high production level of rhBChE, and further application of polysialic acids for capping modification in order to enhance rhBChE stability in the bloodstream.

Focus on current generation of stoichiometric bioscavengers is shifted to modification of recombinant BChE to prolong its stability in the bloodstream. Polyvalent acids may be efficiently used to increase the molecular size of rhBChE. These molecules were shown to improve pharmacokinetics of test drugs leaving the drug active after modification. Unlike other hydrophilic polymers, such as PEG or dextran, polysialic acids are biodegradable, less likely to cause immunogenic response, and their catabolic product (NeuNAc) is not known to be toxic. Thus, chemical polysialylation of cholinesterases and particularly BChE has been

Table 1. Chemical polysialylation has no effect on enzymatic properties and enhances pharmacokinetics of recombinant bioscavenger. Characteristics of non-modified and chemically polysialylated rhBChE-CAO27 compared to hBChE

<table>
<thead>
<tr>
<th>Kinetics</th>
<th>Pharmacokinetics</th>
</tr>
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<tbody>
<tr>
<td>With BTC</td>
<td>VR</td>
</tr>
<tr>
<td>K_m, uM</td>
<td>k_cat per second</td>
</tr>
<tr>
<td>rhBChE</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>rhBChE-CAO27</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>hBChE</td>
<td>23 ± 2</td>
</tr>
</tbody>
</table>

Note that ‘±’ sign states for standard deviation (SD), enzyme kinetic constants (n = 3), inhibition by VR (n = 12).
and bimolecular inhibition constants (kI) compared to unmodified recombinant BChE and naturally-occuring BChE (Table 1) with that it protects mice against 4.2 LD50 of VR exposure administered (equivalent to 400 U) gave an efficacy index score of 4.2, meaning time in bloodstream up to 17 h. A 21 mg/kg dose of rhBChE-CAO27 mice showed that modification with CAO27 enhanced BChE half-

Acetylcholinesterase (AChE) inhibition by organophosphorus compounds (OP) results in the accumulation of neurotransmitter acetylcholine (ACh) and causes several adverse effects, which can eventually lead to death. All OP poisonings call for immediate therapeutic treatment, which usually consists of a combined administration of anticholinergic drugs and an oxime as the reactivator of AChE. However, AChE inhibitors by OP as nerve agent soman is less susceptible to reactivation due to the rapid dealkylation (ageing)(3) of the soman-AChE conjugate. Earlier studies have shown that certain single- and double-site amino acid substitutions in the AChE active center could slow-down ageing and enable oximes to reactivate the soman-AChE conjugate prior to its ageing. Our earlier in vitro studies have uncovered a unique potential of the human AChE mutant, the hY337A/F338A, that combines the increase in the active center accessibility to oximes of the Y337A mutant with the ageing resistance of the F338A mutation. We have proposed that this AChE mutant could be administered to exposed individuals in the form of a pre-treatment to act as pseudo catalytic bioscavenger. Catalytic OP turnover should provide protection by degrading the OP before it reacts with the target AChE. In this study we evaluated in vitro reactivation capacities of several oximes for the soman-inhibited hY337A/F338A. Among the tested oximes, only ICD-585 [1-(3-(4-carbamoylpyridinium-1-yl)propyl)-2-((hydroxyimino)methyl)pyridinium chloride] showed a notable reactivation capability, albeit still smaller than the oxime HI-6, which is currently used in therapy. We furthermore performed an ex vivo study on whole human blood, the source of natural total cholinesterases, combining it with the mutant and either HI-6 or ICD-585 oxime in order to test the mutant’s bioscavenging potential. Indeed, 0.5 μM concentration of soman was decomposed within 25 min when human whole blood was supplemented with 500 nM hY337A/F338A and 1 mM HI-6. These ex vivo findings confirm our earlier in vitro data that hY337A/F338A possesses a true bioscavenging potential when combined with HI-6. The ICD-585 oxime was in turn proven to be promising reactivator of the soman-hY337A/F338A conjugate.

Acknowledgements: We wish to thank Dr Irwin Koplovitz (USAMRICD, USA) for supplying us with ICD-585 oxime. Supported by the NIH (No. U01 NS058046) and Croatian Ministry of Science, Education and Sports (No.022-0222148-2889).

**SW02.W9–13**

Genotyping of single nucleotide polymorphisms of human PON1 and BChE genes by high-resolution DNA melting

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Paraoxonase-1 and butyrylcholinesterase are natural bioscavengers of organophosphate acetylcholinesterase inhibitors in human serum that can determine individual sensitivity to OP toxicity. Inter-individual differences in paraoxonase-1 activity and substrate specificity are strongly associated with two amino-acid substitutions: Leu/Met (L/M) at position 55 (rs854560) and Gin/Arg (Q/R) at position 192 (rs662). Substitution Ala/Thr (A/T) at position 539 in butyrylcholinesterase molecule produces so-called ‘K variant’ of the enzyme (rs1803274). Threonine homozygotes are characterized by reduced activity of BChE. Threonine allele is often co-inherited with atypical butyrylcholinesterase allele (rs1799807). High-resolution DNA melting is a simple, rapid and inexpensive method of genotyping which can be performed in closed-tube format. Hereby the problem of experimental evaluation of applicability of HRM analysis in genotyping of SNPs of human PON1 and BChE is actual. HRMA was performed on CFX96 system (Bio-Rad) using SsoFast EvaGreen Supermix reagent (Bio-Rad), the results were verified by RFLP analysis or Taqman assay.

Genotyping of SNPs rs662 and rs1803274 was facilitated by the nucleotide substitution A>G (G>A), which resulted in changed number of hydrogen bonds in the PCR product and hence in shifted Tm. All three genotypes of each SNP formed separate melt curve clusters.

Genotyping of SNP rs854560 was complicated by the nucleotide substitution T>A, which had no significant effect on Tm of the PCR product. Analysis using short amplicons (59 and 40 bp) failed to discriminate LL and MM homozygotes from each other, melt curves of homozygous samples formed a single cluster. Addition of small quantity of LL homozygote DNA into reaction mixture before PCR possessed discrimination of all three
genotypes due to different amount of heteroduplexes formed in LM and MM samples.

The allelic frequencies (0.660 for L-allele and 0.718 for Q-allele of PON1, 0.766 for A-allele of BChE) obtained from genotyping of 94 habitants of Kirov region were similar to allelic frequencies of Europeans and Caucasian Americans. HRM analysis can be applied for genotyping of human SNP rs662, rs854560 and rs1803274.

SW02.W9–15
Comparative analysis of CID and ETD tandem mass-spectrometry in human serum albumin adductomics
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The development of methods for retrospective detection of exposure to xenobiotics is very important in toxicological analysis. It is known that blood proteins bind and inactivate the agents, thus preventing their toxic effects. In contrast to organophosphorus nerve agents and their hydrolysed metabolites, which often have short half-life in vivo. The corresponding adducts are stable for at least several weeks and, thus, well suited for analysis of biomedical samples.

Interaction of organophosphates with macromolecules in the body is accompanied by the rapid formation of a covalent bond between the phosphorus atom of the organophosphates and the tyrosine residues of albumin and a number of other proteins.

Mass spectrometry is a powerful tool for the identification of proteins and determination of their posttranslational modifications. The aim of this work is the detection and identification of human serum albumin (HSA) adducts with sarin (GB) using CID and ETD fragmentation on LC-MS-MS system (Bruker AmaZon ETD).

In peptic digest of human serum albumin, incubated with sarin were detected two peptides 409-VRYGATKKVPQVST-420 and 408-LVRYGATKKVPQVST-420 of HSA, which are not present in the control sample. Using tandem mass spectrometry with ETD fragmentation amino acid sequences of identified peptides were recovered. Also was established that sarin bind to Y-411. Fragment mass spectrum obtained by CID mode is low informative and does not provide identification of the peptides.

Thus, despite the fact that the CID fragmentation method is widely used in proteomics research in order of identifying post-translational modifications, ETD method is the most preferable.

SW02.W10
Alexander Braunstein Memorial Symposium: Enzymes, Cofactors, Mechanisms (II–W10)

SW02.W10–1
Threonine synthase: role of the product phosphate in determining the reaction pathway
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Threonine synthase (TS) catalyzes the elimination of the γ-phosphate group from O-phospho-L-homoserine (OPHS) and the addition of water at Cβ to generate L-threonine. The reaction mechanism involves all the intermediates known for the PLP enzymes. Therefore, the important question is how TS prevents side reactions and carries out the catalysis specifically.

In the catalysis of Thermus thermophilus HB8 TS (tTS), 98% of OPHS is converted to L-threonine, and 2% to α-ketobutyrate. The branching point of the two reactions is the PLP→α-aminoacrotonate aldimine (AC). When L-vinylglycine was used as the substrate, L-threonine was formed only in the presence of phos-
Sulfate could not substitute phosphate. Transient-kinetic analysis of the reaction of tTS with L-threonine showed that the phosphate ion stabilized the transition state for the hydration at C6 of AC. These observations led us to propose that the phosphate ion released from OPHS remains at the active site of tTS, and acts as the catalyst for the subsequent main reaction steps. However, the details of the catalysis by the phosphate ion, such as whether it acts as the base catalyst for the hydration, were not fully understood.

In order to elucidate the fine catalytic mechanism of this remarkable ‘product-assisted catalysis’, we carried out QM/MM studies on the main and side reactions from AC. The obtained energy profile was in good qualitative agreement with the experimentally determined one, when the PLP N1 was unprotonated and the phosphate ion was monoprotonated. The results showed that Lys61 was the general base catalyst for the nucleophilic attack of a water molecule at C6 and then protonates Cα of AC. The phosphate ion makes a hydrogen bond with the attacking water molecule and possibly stabilizes the transition state for the hydration. Rotation of the Cα–N bond of AC during transdilmination to form α-ketobutyrate destabilizes the transition state by breaking the conjugation of the enamine structure, and this explains why transdilmination proceeds efficiently for L-threonine but not for α-aminoacprotonate.

**SW02.W10–2**

**Sulfur assimilation pathways in bacteria: new avenues for antibiotics**

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The last step of cysteine biosynthesis in bacteria and plants is catalyzed by the PLP-dependent enzyme O-acetylserine sulfhydrylase (OASS). OASS plays a key role in the adaptation of bacteria to the host environment, in the defence mechanisms to oxidative stress and in antibiotic resistance. Because mammals synthesize cysteine from methionine and do not possess OASS, this enzyme is a potential target for antimicrobials [1,2]. In bacteria, two isozymes, OASS-A and OASS-B, have been identified and demonstrated to share similar binding sites [3,4], although the respective roles are still under investigation. A peculiar difference between OASS-A and OASS-B lies in the interaction with serine acetyltransferase (SAT), the preceding enzyme in the biosynthetic pathway. SAT binds and inhibits, via its C-terminal peptide, only OASS-A. By mimicking the natural OASS-A/SAT interaction, we have developed effective pentapeptide inhibitors for both OASS isozymes [2,4]. We have also identified potential inhibitors of OASS-A via classical medicinal chemistry approaches [5]. With the aim of identifying compounds that inhibit specifically either OASS-A or OASS-B or simultaneously both isozymes, a ligand- and structure-based virtual screening of a subset of the ZINC library using FLAP was carried out. The binding affinities of the most promising candidates were measured in vitro on purified OASS-A and OASS-B from Salmonella typhimurium via a direct method that exploits the change in PLP fluorescence. Two molecules were identified with dissociation constants of 3.7 and 33 μM for OASS-A and OASS-B, respectively. Because GRID analysis of the active sites of the two isozymes indicates the presence of some common pharmacophoric features, cross binding titrations were carried out. The best binder for OASS-B binds to OASS-A with a dissociation constant of 29 μM, thus displaying a limited selectivity, whereas the best binder for OASS-A binds OASS-B with a dissociation constant of 50 μM, with an 8-fold selectivity towards the former isozyme. Knowledge of the structural requirements for isosform-specific binding to OASS will allow either to specifically inhibit the isozyme that mainly supports bacteria during infection and long-term survival, or to completely block cysteine biosynthesis using ligands effective on both isozymes.

**References**

**SW02.W10-4**

**Alliinase: structural peculiarities and applying for targeted therapy**


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Alliinase (Cys sulfoxide lyase, alliin lyase; EC 4.4.1.4) from garlic belongs to the class I family of PLP-dependent enzymes. The enzyme is responsible for generation of allicin (diallyl thiosulfinate) a well-known antibotic and cytotoxic agent. For allicin to be effective in vivo it should be produced in situ due to its low stability and short life span. Considering such an approach, alliinase might be a valuable biotechnological tool for the generation of allicin from its precursor alliin (+S-allyl-L-cysteine sulfoxide). Unfortunately, the enzyme reveals a strong tendency to aggregate, and undergoes irreversible inactivation in the presence of denaturants. Alliinase is also fragile and highly folded. Comparison of the apo and holo-enzyme structures reveals that the formation and binding of PLP does not induce a clearly visible conformational change of the enzyme. There is also no shift in the relative positions of the large and small domains. The alliinase active site appears to be predisposed to bind PLP. According to X-ray data the enzyme surface is hydrophilic and its interdomain regions are penetrated by water. We demonstrate here that different osmolytes increased thermostability and prevented inactivation of alliinase. Enhanced stability of alliinase was also found in the naturally occurring stable complex of alliinase with mannose specific garlic lectin ASAI (ALASA) which we were the first to demonstrate. The molar ratio alliinase:ASA-I (A:L) corresponded best to the structure A2L2. Stability of alliinase in the ALASA complex exceeded that of the naked enzyme in the presence of 1M TMAO. The stabilized alliinase was suitable for our novel approach in antifungal therapy using a murine model of invasive pulmonary aspergillosis. We prepared a conjugate consisting of alliinase legated to a monoclonal anti-Aspergillus fumigatus antibody to target the production of allicin molecules to the surface of the fungus. The most impressive therapeutic effect (85% survival at the end of the experiment) was seen with mice that were treated with the conjugate and alliin.

**SW02.W10-5**

**Effects of polymorphic and pathogenic mutations on the structural and functional properties of human alanine:glyoxylate aminotransferase**

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Alanine:glyoxylate aminotransferase (AGT) is a liver-specific peroxisomal pyridoxal 5'-phosphate (PLP)-dependent enzyme belonging to the Fold Type I group. The enzyme catalyses the overall transamination of L-alanine and glyoxylate to pyruvate and glycine; displaying an equilibrium constant of \( K=9400 \). AGT is encoded by the AGXT gene, which is present in human population as two main polymorphic variants termed the major and the minor allele. The latter differs from the major allele mostly by a single-base mutation leading to a P11L amino acid substitution, which generates a putative mitochondrial targeting sequence at the N-terminus. AGT deficiency causes Primary Hyperoxaluria Type I (PH1), a rare inherited disorder characterized by calcium oxalate kidney stones and progressive deposits throughout the body, as a consequence of glyoxylate accumulation and oxidation to oxalate in the cytosol of hepatocytes (1). PH1 is characterized by a strong heterogeneity. In fact, more than 150 pathogenic mutations are known, some of which co-segregate and functionally synergize with the minor allele polymorphism. In the last years, we have carried out an extensive characterization of both normal and polymorphic AGT as well as of several pathogenic variants (2). By combining biochemical analyses on purified proteins with cell biology studies, we highlighted that the molecular mechanisms by which missense mutations cause AGT deficiency include:

- functional defects (loss of catalytic activity, reduced coenzyme binding affinity) as is the case for the G82E and D183N variants
- structural defects (reduced stability, reduced dimerization, aggregation propensity in vitro and/or in the cell), as is the case for G170R, G161S, G161C and P319L

Altogether, the results of our investigations have allowed us to identify the molecular defects of specific variants, thus generating new insights into the pathogenesis of PH1, as well as insights into potential therapeutic strategies for the patients bearing the mutations examined.

**References**


**SW02.W10-6**

**Structural basis of the substrate specificity of human and bacterial kynureninase**

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The kynurenine pathway is the major route for tryptophan catabolism in animals and some fungi and bacteria. Kynureninase catalyzes the hydrolytic cleavage of the \( C_9-C_8 \) bond of L-kynurenine to give anthranilate and L-Ala. The procaryotic enzyme preferentially reacts with L-kynurenine, while eucaryotic kynureninases show preference for 3-hydroxy-L-kynurenine. Crystallography of kynureninases from *Pseudomonas fluorescens* (PfKynase) and *Homo sapiens* (HsKynase) shows that the active sites are nearly identical, except that His-102, Asn-333, and Ser-332 in HsKyn are replaced by Trp-64, Thr-282 and Gly-281 in PfKynase. The structure of HsKynase complexed with an inhibitor, 3-hydroxyhippuric acid, shows that the 3-OH forms an H-bond with the amide carbonyl of Asn-333. Site-directed mutagenesis of HsKyn shows that these residues are, at least in part, responsible for the differences in substrate specificity, since the H102W/S323G/N333T HsKynase triple mutant shows activity with L-kynurenine but not 3-hydroxy-L-kynurenine. Stopped-flow kinetic experiments with PfKynase show that a transient quinonoid intermediate is formed on mixing, which decays to a ketimine at 740 s. Quench experiments show that anilinolate, the first product, is formed in a stoichiometric burst at 50 s, and thus the rate-determining step in the steady state (\( k_{cat}/K=16/s \)) is the release of the second product, L-Ala. \( \beta \)-Benzoylalanine is also a good substrate for PfKyn, but does not show a burst of benzoate formation, indicating that the rate-determining step for this substrate is benzoate release. A Hammett plot of rate constants for substituted \( \beta \)-benzoylalanines is non-linear, suggesting that carbonyl hydration is rate-determining for substrates with electron-donating groups, but \( C_9-C_8 \).
cleavage is rate-determining for substrates with electron-withdrawing groups. The reaction mechanism of Kynase is thus very similar to that proposed by Braunstein in 1949.

**SW02.W10–7**

**Methionine gamma-lyase as a target in pathogens**

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Pyridoxal 5'-phosphate-dependent methionine γ-lyase (EC 4.4.1.11) catalyzes γ-elimination reaction of methionine and its analogs and β-elimination reaction of S-substituted cysteines. The enzyme presents in some bacteria and primitive eukaryotes. It might be a new target in pathogens as mammalian cells do not contain methionine γ-lyase. We performed the search of sequences ascribed to methionine γ-lyase in genomes of pathogenic bacteria. Sequences from genomes of Brucella, Porphyromonas and Clostridium were selected and analyzed. Genes coding methionine γ-lyase in genomes of C. sporogenes, C. tetani and P. gingivalis were cloned, expressed in E. coli cells and substrate specificity of the enzymes was confirmed. As starting molecule for a design of potential inhibitors of the enzyme we studied reaction of the Citrobacter freundii enzyme with non-protein amino acid (2R)-2-amino-3-[[(S)-prop-2-enylsulfinyl]propionic acid (alliin). It was demonstrated that methionine γ-lyase catalyzes the β-elimination reaction of allin with formation of allyl 2-propenethiosulfinate (allicin), which is the best known active compound of garlic. The reaction entails the inactivation of the enzyme in both γ- and β-elimination reactions and the loss of its three SH-groups. Crystal structures of inactivated wild type C. freundii enzyme and its mutant form with the replacement of three SH-groups of methionine γ-lyase were solved (at 1.85 Å resolution). Rational design of the SoyFDH enzyme sharply increased and could achieve up to 9% of total methionine proteins. Bioinformatic analysis showed that many plants have a few genes of FDH.

In this report we will present data about study of two FDH – from Arabidopsis thaliana (AraFDH) and isoenzyme 2 from soya Glycine max (SoyFDH). cDNA of AraFDH and SoyFDH were optimized and cloned in E. coli cells. Both enzymes were expressed as soluble and active proteins with yield up to 1 g of target protein per litre of cultivation medium. Kinetic properties and stability of AraFDH and SoyFDH were studied at different conditions. It was found that plant FDHs show very high resistance to inactivation by hydrogen peroxide which is presented in mitochondria at high concentration under stress conditions. Crystallization of the enzymes were carried out on earth and in space. Crystal structures of apo- and holo-forms were solved with resolution till 1.4 angstrom. Rational design of the SoyFDH resulted in new mutants with higher specific activity, better Michaelis constants and great improvement of thermal stability. Common and characteristic features of properties and structures of microbial and plant FDHs are discussed.

Authors thank Prof. J. Markwell, University of Nebraska (Lincoln, USA) and Prof. N. Labrou, Agricultural University of Athens (Greece) for kind gift of plasmids with full length cDNA of AraFDH and SoyFDH genes, respectively. This work was supported by Russian Foundation for Basic Research (grants 11-04-00920-a and 12-04-31740-mol a).

**SW02.W10–8**

**The intrigues and intricacies of quino-cofactor biosynthesis**

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The enzymatic quino-cofactor family arises via post-translational modifications of aromatic amino acid side chains (tyrosine or tryptophan). These side chain modifications can occur within the active site of the cognate enzyme or via the complex processing of a ribosomally encoded peptide. This presentation will summarize recent structural, genetic and biochemical probes of the generation of the peptide-derived cofactor, pyrroloquinoline quinone (PQQ). Emphasis will be on five essential gene products, their interactions with one another and the necessity of combining anaerobic and aerobic reactions within a single biosynthetic pathway. (Supported by the National Institutes of Health, GM025765).

**SW02.W10–9**

**Plant formate dehydrogenase: structure – function studies**


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NAD⁺-dependent formate dehydrogenase (EC 1.2.1.2, FDH) plays important role in bacteria, yeasts, fungi and plants. In plants FDH is situated in mitochondria in contrast to cytoplasm localization in microorganisms. Plant FDH is the universal protein of stress. Under different stress conditions content of the enzyme sharply increased and could achieve up to 9% of total mitochondria proteins. Bioinformatic analysis showed that many plants have a few genes of FDH.

In this report we will present data about study of two FDH – from Arabidopsis thaliana (AraFDH) and isoenzyme 2 from soya Glycine max (SoyFDH). cDNA of AraFDH and SoyFDH were optimized and cloned in E. coli cells. Both enzymes were expressed as soluble and active proteins with yield up to 1 g of target protein per litre of cultivation medium. Kinetic properties and stability of AraFDH and SoyFDH were studied at different conditions. It was found that plant FDHs show very high resistance to inactivation by hydrogen peroxide which is presented in mitochondria at high concentration under stress conditions. Crystallization of the enzymes were carried out on earth and in space. Crystal structures of apo- and holo-forms were solved with resolution till 1.4 angstrom. Rational design of the SoyFDH resulted in new mutants with higher specific activity, better Michaelis constants and great improvement of thermal stability. Common and characteristic features of properties and structures of microbial and plant FDHs are discussed.

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Plant FDHs show lower values of Michaelis constants both for formate and NAD$^+$ compared to ones from microorganisms. So, such FDHs can be successfully used for cofactor regeneration in fine organic synthesis especially for preparation of chiral compounds. Besides, in plants FDH plays a very important role in stress conditions. Its content dramatically increases during such stress conditions as drought, lack of oxygen, thermal discontinuity, pathogen infections, etc. Therefore, FDHs from plants are worth studying. The object of research in present work was formate dehydrogenase from soya Glycine max. The enzyme has the lowest values of Michaelis constants among even plant FDHs known at the moment.

Rational design approach is successfully used in this laboratory to study structure-function relationship of different enzymes. Analysis of SoyFDH structure surface revealed the Phe residue in 290 position which is localized in coenzyme-binding domain of active site and its replacement may cause changing in enzyme properties. Alignment of FDH amino acid sequences from different sources showed presence of residues Asn, Tyr, Asp, Ser. Computer modeling of influence of different amino acid changes of Phe290 was carried out and the most promising mutations were chosen for future work. Results of modeling shown that some of the Phe290 replacements may cause the formation of additional hydrogen bonds in the enzyme structure. Mutant SoyFDHs with changes of Phe 290 by Asn, Asp, Ser, Tyr, Gln, Gln, Thr and Ala were prepared and purified. Catalytic properties and stability of the mutant enzymes were studied. It was revealed, that practically all mutation influenced on Michaelis constant with formate, but $K_M^{NAD+}$ remained practically unchanged. Thermal stability of mutant SoyFDHs were studied by analysis of inactivation kinetics as well as by differential scanning calorimetry. It was found that mutations of Phe290 resulted also in significant increase of enzyme stability. One of the best enzymes SoyFDH Phe290Asp was 44 times more stable and had Tm 7.8°C higher, then that for wt-SoyFDH.

This work was supported by Russian Foundation for Basic Research (grants 11-04-00959-a and 12-04-31740-mol a).

**SW02.W10–11**

Investigation the role of Met104 in catalytic activity and thermal stability of D-amino acid oxidase

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D-amino acid oxidase is a FAD-containing enzyme catalyzing D-amino acids oxidation to corresponding α-keto acids. DAAO plays important role in regulation of many processes in living cell (especially in mammals). The enzyme is also of high practical interest for pharmaceutical industry, fine organic synthesis and analytical biotechnology. It is used for biocatalytic conversion of cephalosporin C into 7-amino cephalosporanic acid (7-ACA) being the key precursor for synthesis of cephalosporanic antibiotics. D-amino acids oxidase from the yeast Trigonopsis variabilis (TvDAAO) is the most appropriate enzyme for application due to the best properties among all known DAAO’s. Nevertheless wild typeTvDAAO often possesses drawbacks for practical processes. Properties of enzyme can be improved by protein design technique. Here we will present the results of protein engineering of TvDAAO. Computer analysis of TvDAAO structure and docking of substrates to active site revealed flexible amino acid residues, including Met104, located at the entrance and controlling access of substrates to the active site. To clear out the role of Met104 residue in catalysis and thermal stability eight substitutions to small and bulky amino acids were proposed based on computer simulation of potential mutants. Site-directed mutagenesis of Met104 was performed to introduce chosen substitutions. All mutants were obtained in soluble and active form. Study of properties showed that volume of residue in the 104th position has a crucial influence on thermal stability and catalytic activity. So introduction of bulky residues led to significant changes of thermal stability and affected on catalytic efficiency with the most of substrates. To investigate interaction between Met104 and spatially closed Phe54 several double mutants were obtained and characterized.

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**SW02.W10–12**

Glutathione S-transferase alpha from Esox lucius liver: purification and characterization

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Glutathione S-transferases (GST) are one of key enzymes involved in a xenobiotic transformation and protection cells against oxidative stress. GSTs are phase II biotransformation enzymes and are proposed as biomarkers of environmental pollution. In the large family of GST proteins, various isoforms differ significantly in their catalytic properties. In this study, one isoform of a cytosolic glutathione transferase was purified from the liver of freshwater fish pike (Esox lucius) by affinity chromatography. The obtained protein was identified by MALDI-TOF-MS as an alpha class GST, had an isoelectric point of about 6.4 and was composed of two subunits each with a molecular weight of 25235.36 Da. This GST showed significant activity towards mammalian alpha class GSTs substrate 1-chloro-2,4-dinitrobenzene and ethacrynic acid and towards 4-nitroquinoine 1-oxide found to be a preferred substrate of mammalian Mu- and Pi-class GSTs. Kinetic analysis with CDNB as the substrate revealed a $K_m$ of 0.75 mM and $V_{max}$ of 12.81 μM/min per mg of protein. It had maximum activity at a pH 9.0, while in various other organisms most GSTs pH optimum is within neutral values. It also shows a low thermal stability with 80% loss of its initial activity at 45°C for 20 min. A comparison with higher vertebrates and some fish species GSTs suggests that the purified protein in general is a typical member of the family of cytosol glutathione S-transferases.
considering aspects like cell permeability, which allows extracellular administration of the cofactor, and the possibility to track the enzyme's product.

The key candidate of our investigation is DNA methyltransferases, that use S-adenosyl methionine (SAM) as methyl donor to methylate specific DNA target sequences. Although these enzymes are epigenetic mediators, their genomic targets are often unknown and their cellular remain poorly understood. To remodel the catalytic site for the new cofactor, a protein engineering study has been carried out, using computational design as well as directed evolution. We believe that the evolved mammalian DNA methylases which have acquired orthogonality for the synthetic cofactor, as well as the ability to modify DNA with tractable groups, will provide new insights regarding the role of this enzyme in epigenetics, including in dictating the genomic methylation patterns in cancer.

**SW02.W10–14**

**Cytochrome c oxidase activity and its subunits gene expression in white muscles of fish: age and season-related changes**

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Cytochrome c oxidase (COX) is the terminal enzyme of the electron transport chain of mitochondria. The regulation of COX activity is complicated due to its complex structure. COX is composed of 13 subunits; three of them are catalytic and encoded by mtDNA and 10 are encoded by nuclear genes. It is known that nuclear subunits take part in regulation of enzyme activity and its tissue specificity. In connection with functional differentiation of COX subunits, the study of the mechanisms of enzyme activity regulation on the level of gene expression of its subunits is of great importance.

The first aspect of our research is devoted to regulation of COX activity in white muscle of fish in ontogenesis. We measured the maximal activity of cytochrome c oxidase and transcript levels of mitochondrial subunit Cox1 and nuclear Cox4 in skeletal muscle of salmon (Salmo Salar L.) of different ages (0+, 1+, 2+). The second aspect is devoted to regulation of COX activity with regard to different seasons. Induction of mitochondrial biogenesis along with seasonal temperature decrease, reflected in increase in COX activity has been documented in many sh species, yet little is known about how this process is reflected in increase in COX activity with regard to different seasons. Induction of mitochondrial biogenesis along with seasonal temperature decrease, reflected in increase in COX activity has been documented in many fish species, yet little is known about how this process is reflected in increase in COX activity with regard to different seasons. Our results show that the expression of COX subunits, particularly, Cox4, is regulated. We assessed the activity of cytochrome c oxidase and mRNA levels of subunits Cox1 and Cox4 in skeletal muscle of rainbow trout collected in summer, fall and winter. Our results suggest that age and season-related COX activity changes is at least partially determined by the regulation on the level of nuclear subunits (particularly, Cox4) expression.

The research was carried out using the facilities of the Equipment Sharing Centre of the Institute of Biology, KarRC of RAS. The research was supported by grant of the Russian Federation President ‘Leading scientific school of Russia’ 1642.2012.4; grant of the Russian Foundation for Basic Research № 11-04-00167_a; the Federal Purposive Program (FCP, state contract № 8050), the Presidential of RAS Programme ‘The Living Nature’.

**SW02.W10–15**

**S250F variant associated with aromatic amino acid decarboxylase deficiency: molecular defects and intracellular rescue by pyridoxine**

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Dopa or aromatic amino acid decarboxylase (DDC, AADC) is a pyridoxal S-phosphate-dependent enzyme that catalyzes the production of the neurotransmitters dopamine and serotonin. Among the so far identified mutations associated with AADC deficiency, an inherited rare neurometabolic disease, the S250F mutation is the most frequent [1–3]. Here, for the first time, the molecular basis for the functional deficit of the S250F variant was investigated both ‘in vitro’ and in cellular systems. Although Ser at position 250 is not essential for the catalytic activity of the enzyme, its mutation to Phe causes a approximately seven-fold reduction of catalytic efficiency and a conformational change in the proximity of the mutated residue transmitted to the active site. In cellular extracts of E. coli and mammalian cells, both the specific activity and the protein level of the variant decrease with respect to the wild-type. The results with mammalian cells indicate that the mutation does not affect intracellular mRNA levels and are consistent with a model where S250F undergoes a degradation process via the proteasome, possibly by the ubiquitin system, occurring faster than the wild-type. Overall, biochemical and cell biology experiments show that loss of function of S250F occurs by two distinct but not exclusive mechanisms affecting activity and folding. Importantly, exposure of mutant-expressing cells to 4-phenylbutirric acid (4-PBA) or, to a major extent, pyridoxine increases the expression level and, in a dose-dependent manner, the specific activity. This strongly suggests that 4-PBA and/or pyridoxine administration may be of important value in therapy of patients bearing S250F mutation.

**References**


**SW02.W10–16**

**Modulation of protein and mRNA expressions of rat liver vitamin D3 metabolizing CYP27B1 by o-coumaric acid**

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CYP27B1 is the member of cytochrome P450 super-family and one of the most important enzymes involved in vitamin D3 metabolism. CYP27B1 is a mitochondrial enzyme and also known as 25-hydroxyvitamin D3 1alpha-hydroxylase since it involves in 1α-hydroxylation of 25-OH-D3 into 1,25-(OH)2D3.
Phenolic compounds are biologically active molecules and suggested to have some disease-preventive properties especially in cardiovascular diseases and certain types of cancer. o-Coumaric acid has been known as a phenolic compound and found in a wide variety of plants such as peanuts, tomatoes, carrots, and garlic. It functions as antioxidant and chemopreventive agents.

In this study, in vivo effects of o-coumaric acid on rat liver CYP27B1 mRNA and protein expressions were investigated. To achieve this goal, 30 mg o-coumaric acid/kg body weight/day was intraperitoneally injected to 14 albino Wistar rats while eight rats were used as control. After 9 days of injection, the rats were decapitated and SI.5 fractions of livers were prepared by homogenization and centrifugation. Effects of o-coumaric acid on rat liver mRNA and protein expressions were analyzed by qRT-PCR and western blotting techniques, respectively. Injection of o-coumaric acid to rats caused 1.85 fold increase in mRNA expression of CYP27B1 with respect to controls and normalized with GAPDH expression as an internal reference (p < 0.05). On the other hand, o-coumaric acid treatment caused 15.52% decrease in CYP27B1 protein expression (p < 0.05).

In conclusion, the vitamin D3 metabolism may be altered due to the changes in mRNA and protein expressions of CYP27B1 by the treatment of rats with o-coumaric acid.

SW02.W10–17
Crystal structures modeling two elementary stages of gamma-eliminations reaction catalyzed by Citrobacter freundii methionine gamma-lyase
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Methionine γ-lyase (MGL, EC 4.4.1.11) catalyzes pyridoxal-5-phosphate-dependent γ-elimination reaction of L-methionine to give ketobutyric acid, methanethiol, and ammonia. MGL has been found in a number of bacteria, some of which are anaerobic parasites. It has been found to be an effective anti-tumor agent in vitro and in vivo. Thus the enzyme is a promising target in some anaerobic pathogens and possible antitumor medicine.

Crystal structures of the enzyme complexed with two competitive inhibitors, L-norleucine and glycine and with substrate, L-vinylglycine were solved at 1.63 Å resolution. The structure of the enzyme complexed with L-vinylglycine allowed to propose that side chain of Lys210 moves as a diaza abstracting the proton from C4-atom of β,γ-unsaturated ketimine intermediate and transferring it to C7-atom with formation of aminoaacrylate intermediate. This residue accomplishes the γ-elimination reaction by the nucleophilic attack to C4-atom of aminoaacrylate which leads to a liberation of products and a regeneration of the holoenzyme.

SW02.W10–18
The thioredoxin system in Streptococcus mutans and Streptococcus thermophilus: an insight on molecular and functional characterization of protein components
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The Streptococcus genus includes several pathogenic species, such as S. mutans, the main responsible of dental caries, and the non pathogen S. thermophilus, used for the manufacture of dairy products. These fermenting bacteria are facultative anaerobes, as they tolerate moderate oxygen concentrations; however, Streptococci possess some crucial enzymes for the defence against ROS. The thioredoxin system is the key element for repairing cellular damages caused by ROS, as it preserves the reduced state of cytosolic proteins. This work addresses the molecular and functional characterization of the thioredoxin components in S. mutans and S. thermophilus, to evaluate how this repairing system works in these sources. Usually, the thioredoxin system is composed by the flavoenzyme thioredoxin reductase (TrxB) and its natural substrate thioredoxin (TrxA) and uses NADPH as electron donor. The redundant putative genes encoding TrxB and TrxA in the S. mutans and S. thermophilus genome were analysed and the corresponding recombinant proteins were purified. In particular, a single TrxB was obtained from either S. mutans (SmTrxB) or S. thermophilus (StTrxB), whereas two TrxA were prepared from either S. mutans (SmTrxA and SmTrxH1) or S. thermophilus (StTrxA1 and StTrxA2). The functionality of the recombinant enzymes was tested through specific biochemical assays. Indeed, both SmTrxB and StTrxB reduced the synthetic substrate DTNB in the presence of NADPH; conversely, among the four streptococcal thioredoxins, only SmTrxA and StTrxA1 accelerated the insulin reduction in the presence of DTT. The combined activity of the streptococcal thioredoxin components was tested through the insulin precipitation in the absence of DTT, in order to reconstitute the thioredoxin system. The assay is functioning with the combination of SmTrxB and StTrxB with either SmTrxA or SmTrxH1. These results suggest that the streptococcal members of the thioredoxin system display a direct functional interaction between them and that these protein components are interchangeable within the Streptococcus genus. In conclusion, our data prove the existence of a functioning thioredoxin system even in these microaerophiles.

SW02.W10–19
Partial purification and characterization of a novel debranching enzyme from Globe Artichoke (Cynara Scolymus-L.)
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Pullulanase is a debranching enzyme which hydrolyses the α-1,6-glucosidic linkages in pullulan and other amyloitic polysaccharides and belong to a family 13-glycosyl hydrolyses, also called the α-amylase family. The affinity of debranching enzymes for...
Having a Km value of 9.14

Centrifuged at the same conditions. This step resulted in a 2.45

Pre-equilibrated with 5% SDS and the extracting buffer and then

The extract (6 ml) was then applied to a Vivaspin 6 ultrafiltration

Through muslin and centrifuged at 10 000 rpm at 4

Phosphate buffer in Waring Blender. The extract was filtered

Material was extracted with 100 ml of 100 mM pH 6.5 sodium-

Ethanol and n-butanol were found to be the strongest inhibitors

Found to be 35

In baking industry as the antistaling agent to improve texture,

Minor application in the manufacturing of low caloric beer and

In antiquing industry as to improve texture, volume and flavor of bakery products [1–3].

In this study, the debranching enzyme was studied for the first
time at globe artichoke. The enzyme was partially purified from
the leaves of the head part of the plant. Ten gram of the plant
material was extracted with 100 ml of 100 mM pH 6.5 sodium-
phosphate buffer in Waring Blender. The extract was filtered
through muslin and centrifuged at 10 000 rpm at 4°C for 1 h.
The extract (6 ml) was then applied to a Vivaspin 6 ultrafiltration
membrane unit, made of polyethersulfone, having 5000 MWCO,
pre-equilibrated with 5% SDS and the extracting buffer and then
centrifuged at the same conditions. This step resulted in a 2.45
fold purification of the enzyme. The partially purified enzyme
showed catalytic activity towards pullulan, dextrin, amylopectin
and starch. Pullulan seemed to be the most suitable substrate
having a Km value of 9.14 × 10^-4 mg/ml and Vmax of 0.2612 U/
ml. The optimum pH for the debranching enzyme was between
5.0 and 7.0 [pH 5.0 for starch and dextrin, pH 6.0 for amylopectin
and pH 7.0 for pullulan]. The optimal temperature was found to be 35°C for pullulan, amylopectin and starch where it was 45°C for dextrin. The enzyme activity was strongly stimulated
by CoCl2, MnSO4, MgSO4 and LiSO4, CH3COOH, NH4,
ethanol and n-butanol were found to be the strongest inhibitors
of the enzyme.

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**SW02.W10–20**

**Pre-steady-state kinetics of substrate recognition and processing by Citrobacter freundii methionine gamma-lyase**

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Methionine γ-lyase (EC 4.4.1.11, MGL) is a pyridoxal 5’-phosphate dependent enzyme found in some bacteria, primitive protozoa etc. MGL catalyzes the irreversible γ-elimination of L-methionine to give methanethiol, α-ketobutyrate and ammonia. The enzyme also catalyzes β-elimination reaction of L-cysteine and S-substituted L-cysteines and γ- and β-replacement reactions of L-methionine and L-cysteine and their analogs. The absence of this enzyme in mammals allows to consider it as a biochemical target for a design of new antipathogenic agents. For this purpose, elucidation of MGL mechanism is crucial. Many its features remain poorly explained despite the availability of structural information on some enzyme’s complexes.

In this study, we analyzed the MGL catalytic mechanism of γ-elimination reaction using stopped-flow kinetics with single-wave-length absorbance detection of transient intermediates. The interaction of MGL with L-methionine (Met), L-vinylglycine (Gly (vinyl)), L-methionine sulfone (MetO2) and O-acetyl-L-homoserine (Hse-OAc) have been studied using registration of absorbance at 420, 320 and 480 nm. The absorbance time-course curves were different for all substrates. The kinetic scheme was proposed and the intrinsic rate constants of main steps of γ-elimination reaction were determined using a global fitting procedure. The obtained data are important for understanding characteristics of elementary stages of γ-elimination reaction.

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**SW02.W10–21**

**Modulation of D-serine cellular concentration by human D-amino acid oxidase**

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D-Serine is a brain enriched transmitter-like molecule that activates NMDA receptors, playing a main role in neuron-glia communication and excitatory neurotransmission. D-Ser is synthesized by serine racemase and degraded by the same enzyme and/or by D-amino acid oxidase (DAO, EC 1.4.3.3). The FAD-containing DAAO has played a prominent role in the development of present concepts in mechanistic flavin enzymology.

In 2002, genetic evidences in humans indicated thatDAO – and its negative regulator pLG72 – are related to schizophrenia (SZ) susceptibility. We proposed a model to explain the DAAO/ pLG72/D-Ser association with the onset of the pathology: hypoactivation of NMDA receptors, finally leading to hypoactivation of NMDA receptors.
hDAAO shows peculiar biochemical properties that distinguish it from known DAAOs; it is a stable homodimer, weakly binds the cofactor, and $K_m$ for D-Ser is above the physiological concentration of the neurotransmitter. Indeed, D-Ser cellular concentration depends on the expression of active hDAAO. By using U87 glioblastoma cells expressing EYFP-DAAO and/or pLG72-ECFP proteins, we provided evidence that newly synthesized hDAAO is located in the cytosol where it can interact with pLG72, placed on the external mitochondrial membrane. The largest part of hDAAO is degraded by the lysosomal system while pLG72 (a protein showing a rapid turnover) is mainly targeted to the proteasome: pLG72 binding increases the degradation of hDAAO (a long-lived protein). Since neosynthesized cytosolic hDAAO is active, pLG72 binding to hDAAO might play a protective role against excessive D-Ser depletion. Finally, we investigated the effect of SNPs in hDAAO related to SZ susceptibility. In agreement with the observed increased activity, we found that these substitutions could negatively affect cellular D-Ser concentration and thus might be relevant for SZ susceptibility.

Our results uncover basic molecular aspects of the D-Ser catabolism and open novel perspective for therapeutic strategies of SZ by targeting hDAAO.

**SW02.W10–23**

**In vitro investigation of HMG-CoA reductase inhibitory effects of various coumarin derivatives**

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Abnormal levels of lipids and/or lipoproteins in blood have been identified as a primary risk factor for cardiovascular diseases (CVD), which are the leading cause of death in men and women older than 65 years. The control of cholesterol blood level is of considerable interest for the therapeutic control of coronary diseases. Current therapeutic strategies are generally directed at lowering serum Low Density Lipoprotein (LDL) cholesterol levels.

Cholesterol homeostasis is regulated by both the receptor mediated uptake of plasma LDL by LDL-receptors and within cells by de novo cholesterol synthesis. The rate-limiting enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG) catalyzes the conversion of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) to mevalonate. The strategies used to reduce serum cholesterol levels are based on the inhibition of HMGR activity and the consequent increase of LDL-receptor membrane exposure. So HMGR is the target of compounds like statins which are very effective in lowering serum cholesterol levels. Statins are usually well tolerated but side effects that concern skeletal muscle may arise; such as increases in creatine kinase, muscle pain and cramps, myositis and rare but potentially life threatening rhabdomyolysis. However, despite their therapeutic efficiencies, statins have not entirely eliminated the risk posed by CVD. Thus, alternative physiological strategies to further lower LDL-C levels effectively and safely are being actively sought.

In recent years many coumarins and their derivatives displaying various pharmacological activities (anti-inflammatory, analgesic, anti-epileptic, anti-atherosclerotic, diuretic and antioxidant) are very popular and have also potential to lower plasma lipid levels. In this study, pravastatin and five different coumarin derivatives that we have synthesized in our laboratory were screened for their abilities to inhibit the catalytic activity of human recombinant HMGR *in vitro*. The most efficient inhibition was obtained with 7,8-dihydroxy-3-(4-methylphenyl) coumarin. A moderate inhibition of HMGR was observed for all the coumarin derivatives tested but was not potent as pravastatin when compared.

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**SW02.W10–23**

**Computer simulations of the structures of horseradish peroxidase with chemically modified prosthetic group**

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Today, there are several approaches to obtaining biocatalysts with desired properties. Site-directed mutagenesis is most commonly used for this purpose. But another powerful method for study and modulation of macromolecular function is chemical modification of proteins. Sometimes, its use allows producing enzymes with the properties that can’t be obtained using genetic engineering. In practice, such products can be used in different areas of biotechnology.

Horseradish peroxidase (HRP) is one of the most studied heme-containing enzymes of the plant peroxidase superfamily and it is the biocatalyst of high practical interest. HRP is widely used in different bioanalytical applications and diagnostic kits. It was reported that catalytic activity and stability of HRP in aqueous buffer and organic media can be greatly improved through the technique of chemical modification of lysine residues. However, this approach is non-specific in contrast to the replacement of native heme with the artificial prosthetic group (APG). Experiments on the production of reconstructed HRP with modified heme demonstrated that APG incorporation into the apoenzyme efficiently proceeds even when bulky substituents are used for the modification of the heme propionate groups. Moreover, reconstructed HRP had improved catalytic activity, increased thermostability and solvent tolerance. Thus, HRP properties can be modulated by variation of substituents introduced into heme.

In order to understand the mechanism and effects of changes in the properties of HRP with modified heme derivatives, we performed computer simulations of the three-dimensional structures of the apoenzyme-modified heme complexes. The computer simulation results for the structure of HRP with chemically modified heme are in a good agreement with the experimental data. Thus, computer simulations can be used to design experiments for heme modification. Thereby, we have designed several APGs. Computer models for the APG–apoenzyme complexes were obtained. Based on results of computer simulations we have selected a few APGs for following chemical synthesis. The experiments on the reconstitution of apo-HRP with APGs are in progress.

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**SW02.W10–24**

**Preliminary identification of proteins that interact with acyl-CoA synthetase family member 4 (ACSF4)**

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**Background:** Acyl-CoA Synthase Family Member 4 (ACSF4, UniProt Q4L235) is the only vertebrate protein showing sequence similarity to microbial nonribosomal peptide synthetases. Since ACSF4 also resembles the yeast 2-aminoadipate reductase (P07702), it was postulated to be a 2-aminoadipate-semialdehyde dehydrogenase (AASDH) that catalyzes the oxidation of 2-aminoadipate 6-semialdehyde (AAS) to 2-aminoadipate (AA) in the L-lysine degradation pathway in mammals. However, ACSF4-dependent oxidation of AAS has never been confirmed and AASDH was identified as Antiquitin protein (P49419) in mammals indicating that the role of ACSF4 remains unknown.

**Objective:** ACSF4 contains adenylation, thiolation and WD-repeat domains in its structure. While the presence of activation domains suggests that a low molecular weight compound may be a substrate for this enzyme, the WD domain appears to coordinate protein-protein interactions. Thus, identification of ACSF4 interactors might help to disclose the biological role of ACSF4 and it was the aim of this investigation.

**Methods:** The cDNA of ACSF4 was amplified from mouse muscle and cloned into the pEF6/HisB vector, which allows the production of proteins with an N-terminal HisTag. The enzyme was overexpressed in HEK 293T cells and purified by nickel-agarose affinity chromatography. Recombinant ACSF4 was bound to anti-HisTag antibody coupled to protein A/G agarose and used as a bait to screen lysates of mouse HEPA 1-6 and human HEK293T and K562 cells. After washing out non-specifically bound proteins the preys were eluted, analyzed by SDS-PAGE and identified by MS/MS.

**Results and Conclusion:** MS/MS analysis revealed the presence of 30–70 proteins in eluted fractions. SSA27 (P56753) was identified as a potential interactor in mouse HEPA lysates and DCAF7 (P61962) and LSM12 (Q3MHD2) were the only proteins of both mouse and human cells that were exclusively found in samples containing recombinant ACSF4. In view of these data, identified proteins may interact with ACSF4, however, the importance of these interactions remains unresolved.

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**SW02.W10–25**

**Concerted protein–protein interactions drive heme delivery in cytochrome c assembly**


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Cytochromes c are a diverse and widespread family of proteins that are central to the life of most organisms. They contain heme covalently bound to the polypeptide in a highly conserved and stereospecific way. In many bacteria and in certain mitochondria, the synthesis of cytochromes c is performed by a complex post-translational modification apparatus called Cytochrome c maturation (Ccm) system comprising eight maturation proteins and a variety of assisting partners. Several Ccm proteins are involved in heme handling but the mechanism of heme transfer from one protein to the next is not known. We used extensive site-directed mutagenesis in an in vivo experimental setup, in conjunction with state-of-the-art mass spectrometry methods, to track the heme from its arrival to the periplasm to its delivery to the cytochrome c substrate.

It has been proposed that heme binds covalently to CcmE, the central protein of the maturation apparatus, before it is transferred to the cytochrome. We isolated and characterised a covalent, ternary complex between the heme chaperone, heme and the cytochrome substrate. Formation of this complex occurs in vivo and in vitro and the amounts of complex produced correlate, in a functionally relevant way, to mutations in proteins of the maturation system. This trapped catalytic intermediate proves that apocytochrome acquires its cofactor directly from CcmE in a pivotal step of the maturation pathway. We also investigated the route for delivery of heme to CcmE and the role of the remaining maturation proteins in the formation and resolution of the CcmE-heme-cytochrome complex. We used a variant form of the classical Ccm system, found in archaea and sulfate-reducing bacteria. Differences in the heme chemistry involved in formation of holoCcmE and holocytochrome in the variant system allowed us to probe the function of the individual component proteins and their concerted action, in transferring heme between the different maturation proteins and finally to cytochrome c.

Cytochrome c assembly seems to be driven by protein–protein interactions. It is a tale of an ensemble of proteins which source and protect the otherwise toxic heme cofactor while providing the appropriate reaction environment for this complex post-translational modification.

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**SW02.W10–26**

**Characterization of the first extremophilic levansucrase purified from halophilic bacteria Halomonas smyrnensis AAD6T**

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Levan is a good candidate as a microbial polymer for many commercial applications in different industrial sectors like food, petroleum, cosmetics and pharmaceuticals [Kazak et al., 2010]. Particularly, being anticarcinogenic, immuno-stimulating and low caloric, make levan more favorable for applications in medical industry [Belghith et al., 2012]. Levan polymer is formed as an extracellular polysaccharide by levansucrase (EC 2.4.1.10) which catalyzes both sucrose hydrolysis and the polymerization of fructose units. Previous studies indicated that The halophilic Halomonas smyrnensis strain AAD6 [Poli et al., 2013] employs a fructosyltransferase (FTF) to synthesize a fructose polymer (a fructan of the levan type, with fructosyltransferase (FTF) to synthesize a fructose polymer (a fructan of the levan type, with (b(2–6) linkages) from sucrose [Poli et al. 2009]. Recent study on the whole genome sequencing of H. smyrnensis strains AAD6 [Poli et al., 2013] employs a fructosyltransferase (FTF) to synthesize a fructose polymer (a fructan of the levan type, with (b(2–6) linkages) from sucrose [Poli et al. 2009]. Recent study on the whole genome sequencing of H. smyrnensis strain AAD6 indicated the presence of a single levansucrase-encoding gene in strain AAD6: sacB [Sogutcu et al. 2012]. In the present study, a downstream processing strategy to recover the enzyme from the fermentation broth was designed and optimized. Sucrose inducible levansucrase by Halomonas smyrnensis is unique in its ability to exhibit catalytic activity at a wide range of salinities due to the halophilic nature of the producer strain. Therefore, by changing the salinity conditions, a very effective and fast protocol was developed and applied. The purification of this levansucrase and identification of peptide amino acid sequences, allowed isolation of the first extremophilic...
levanucrase gene (\textit{secB}), encoding a protein consisting of 416 amino acids with a molecular size of 46.22 kDa. The encoded protein showed highest similarity with levanucrase (Beta-D-fructofuranosyl transferase; sucrose 6-fructosyl transferase) from \textit{Arsenophonus nasoniae}. Studies on investigation of the dependence of the hydrolysis and transferase activities of the enzyme total temperature, pH, salinity, and metal ions (especially Fe^{2+}) are still continuing. These findings may give new possibilities in elucidating the kinetics of the mechanism of the levanucrase enzyme by \textit{Halomonas snyrensis}. This research has been supported by TUBITAK (MAG/110M613).

**References**


**SW02.W10–27**

\textbf{Allosteric mechanism for the regulation of the activities of Bacillus subtilis phosphoribosyl pyrophosphate synthetase}

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Phosphoribosyl pyrophosphate synthetase (PRS, EC 2.7.6.1) catalyzes the reaction of ATP with ribose 5-phosphate (R5P) to form AMP and phosphoribosyl pyrophosphate. \textit{Bacillus subtilis} PRS (bsPRS) belong to class I, which require Mg^{2+} and phosphate for enzymatic activity, but is inhibited allosterically by ADP. To investigate the allosteric regulation of bsPRS, we have cloned, expressed and purified bsPRS in \textit{E. coli}. The activity was measured by a coupled assay with adenylate kinase, pyruvate kinase, and lactate dehydrogenase to couple the formation of the product AMP to the conversion of NADH to NAD^{+}. Steady-state kinetic study reveals that both of phosphate and sulfate bind at the same regulatory site and act as V-type heterotropic allosteric effectors for bsPRS. In the comparison of the activation by phosphate, sulfate decreases the \textit{k}_{cat} and \textit{k}_{cat}/\textit{K}_{m} by two-fold and 6.5-fold respectively, and increases the activation constant (\textit{K}_{act}) by three-fold. In addition, both phosphate and sulfate increase the catalytic efficiency for Mg^{2+} by 110- and 22-fold, respectively. In the absence of activator, phosphate, the kinetics of bsPRS shows a complete positive cooperativity for MgATP and negative cooperativity for R5P. By increasing the concentrations of R5P, phosphate and sulfate, the conformation of bsPRS shifts to R state, decreasing the cooperativity for MgATP. These results suggest that phosphate or sulfate behaviors as a heterotropic allosteric effector stabilizing the R-form and a substrate induced conformational changes of bsPRS, where R5P binds well to E-MgATP complex (non-cooperative), but binding of the next is more difficult (negative cooperativity).

**SW02.W10–28**

\textbf{Kinetic parameters and cytotoxic activity of recombinant methionine gamma-lyase from pathogenic sources}

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Methionine starvation can broadly affect protein synthesis and modulate gene methylation, cell cycle transition, and pathways related to survival following DNA damage in tumor but not in normal tissues. The utilization of methionine depleting enzyme methionine \gamma-lyase (MGL) as possible anticancer agent seems to be very promising. Recently it was demonstrated that recombinant enzyme from \textit{Pseudomonas putida} could effectively inhibit broad series of cancer cell lines. However, \textit{P. putida} MGL is unstable in serum (t_{1/2} = 1.9 \pm 0.2 h), in contrast to a half-life of 4 h for a comparable dose of the same enzyme from pathogenic bacteria \textit{Clostridium sporogenes}.

The genes of the MGL from three pathogenic bacteria \textit{C. sporogenes}, \textit{Clostridium tetani}, \textit{Porphyromonas gingivalis} were cloned and expressed in \textit{E. coli} cells; homogenous preparations of the enzyme were obtained, and steady-state kinetic parameters of the recombinant MGL from these sources were determined in \beta- and \gamma-elimination reactions. The enzyme from \textit{C. sporogenes} has the most catalytic efficiency in \gamma-elimination reaction of methionine.

The cytotoxicity of MGL from \textit{C. sporogenes}, \textit{C. tetani} and \textit{Citrobacter freundii} against K562, PC-3, LnCap, MCF7, SKOV-3, L5178y tumor cell lines was evaluated. The most sensitive cell lines were: K562 (IC50 = 0.4–1.3 U/ml); PC-3 (IC50 = 0.1–0.4 U/ml) and MCF7 (IC50 = 0.04–3.2 U/ml).

**Acknowledgements:**

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SW02.W10–30  
Connecting known and new metabolic pathways related to purine bases catabolism through a transcriptomic analysis of the soil bacterium Acinetobacter baylyi ADP1

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Bacterial degradation of various purine bases and derivatives for nutritional purposes proceeds through uric acid as an intermediate. This is the case for the cognate adenine and guanine found in nucleic acids and for modified and damaged bases like 7-methylguanine and 8-oxoguanine. It has been also reported that the vitamin components pteridines without further details up to now. In this work, we identified known and new metabolic pathways connected to uric acid catabolism by using a RNA-seq approach in the gram-negative, naturally highly competent for DNA transformation and nutritionally versatile soil bacterium Acinetobacter baylyi ADP1, for which tools like a complete deletion mutant strain collection and an ORFeome have been developed in our laboratory. Comparison of transcripts levels of cultures grown till mid log-phase with uric acid versus ammonium as a nitrogen source revealed that half of the 3300 genes differen- tially expressed, with 70 genes being highly upregulated (log2-Fold change >2, up to 250 fold increase in transcript abundance) and 300 genes highly downregulated (log2Fold change less than −2, up to 400 times decrease of transcript abundance; half of the genes are of unknown function). The overexpressed genes encompass at least 16 annotated transporters, 10 oxidoreductases, and four hydrolases. Most of them clustered in discrete groups of genes that are functionally related in other genomes, indicating a possible metabolic link. The pathway leading from uric acid to urea was clearly induced, but not at the highest level. Although the function of some clusters still remains elusive, we now provide candidate genes for the catabolism of alkylated purines and pteridines by combining the analysis of this transcriptomic response to uric acid and the growth profiles of our mutant collection. Based on their functional annotation, the enzymes catalyzing oxidative steps will be characterized in vitro.

Abstracts SW02 Biocatalytic Mechanisms and Protein Dynamics  

SW02.W10–31  
Molecular basis of control of endothelial and neuronal NO synthase

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Nitric oxide produced by endothelial NO synthase (eNOS) is a critical signal in cardiovascular function and a factor in related areas such as diabetes associated vascular disease. Neuronal NO synthase (nNOS) produces NO as a retrograde neurotransmitter. Regulation includes inputs from phosphorylation, protein-protein interactions, and associated localization of eNOS as well as calmodulin (CaM) mediated control. Primary control is exerted through regulation of electron flux from NADPH to the oxygenase active site; tethered shuttle mechanisms for eNOS electron transfer posit alternate input and output conformations. Crystal structures of the ‘input state’ show FMN in close contact with FAD but remote from solute; epr shows in addition an ‘output state’ with heme-FMN radical coupling.

Recently we showed that mammalian NOS isoforms have multiple conformational states. In eNOS, but not nNOS, this state arose through indeterminate state with a conformational effect. The ‘input state’ show FMN in close contact with FAD but remote from solute; epr shows in addition an ‘output state’ with heme-FMN radical coupling.

Two major control modes are evident in eNOS and nNOS. In direct control, protein- protein interactions (e.g., CaM binding) or covalent modification (e.g., phosphorylation) affect the ability of NO to move through obligatory conformational intermediates. In indirect control, protein binding or phosphorylation increase or decrease the effects of direct regulators.
SW02.W10–32

Active site residues Tyr58, Tyr113 and Ser339 of Citrobacter freundii methionine gamma-lyase: the role of the hydroxyl groups in the reaction mechanism

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Citrobacter freundii methionine γ-lyase (MGL, EC 4.4.1.11) is a homotetrameric pyridoxal 5’-phosphate-dependent enzyme catalyzing the γ-elimination and γ-replacement reactions of L-methionine and its analogs as well as β-elimination and β-replacement reactions of L-cysteine and S-substituted L-cysteines. The enzyme has been found in many pathogenic microorganisms and absent in mammalian cells. Thus MGL might be considered as an attractive target in pathogens. Three-dimensional structure of Citrobacter freundii MGL revealed a number of active site residues. Mutant forms of MGL with replacements of active site residues Tyr58, Tyr113 and Ser339 were obtained by site-directed mutagenesis. High quality crystals of the mutant forms were obtained. Structural analyses of the enzymes Tyr58Phe, Tyr113Phe and Ser339Ala have been solved. Comparison of spatial structures of the wild type holoenzyme and the mutant forms demonstrated that the replacements did not result in essential changes of the conformation of the active sites of mutant enzymes. Spectral and kinetic characteristics of the mutant enzymes were studied. The catalytic efficiencies in both β- and γ-elimination reactions decreased for about 2-orders of magnitude for the mutant enzymes, and only in γ-elimination reaction for the enzyme Tyr113Phe the catalytic efficiency decreased for about 4-orders of magnitude as compared to those for the wild type enzyme. Spectral data on the mutant forms complexed with the substrates and inhibitors demonstrated that the replacements led to the changes of equilibrium distributions of species in the complexes as compared to those for the wild type enzyme. One may assume that Tyr58 and Ser339 might be involved in stabilization of the catalytic residue Lys210 at some stages of the enzymatic reaction and Tyr113 might be a general acid catalyst at the stage of methylthiol elimination.

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SW02.W10–33

Potential role of cytosolic 5’-nucleotidases in human NAD metabolism

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Nicotinamide adenine dinucleotide (NAD) is an essential electron carrier in redox reactions. Moreover, NAD is used as a substrate and is degraded by several classes of enzymes including ADP-ribose transferases, poly(ADP ribose)polymerases, protein deacetylases (sirtuins) and ADP-ribosyl cyclases. These NAD-dependent enzymes control signaling processes, thereby regulating gene expression, cell cycle progression, DNA repair, apoptosis, aging and others. To ensure proper control of vital reactions, NAD must be permanently resynthesized. NAD synthesis starts from vitamins delivered with food: nicotinamide and nicotinic acid as well as nicotinamide riboside (NR) and nicotinic acid riboside (NAR). These ribosides play an important role in NAD metabolism. For example, NR and NAR are authentic intracellular metabolites in yeast and can be generated by dephosphorylation of nicotinamide mononucleotide (NMN) and nicotinic acid mononucleotide (NAMN). How NR and NAR are generated in human cells is unknown. In this work we have tested whether human cytosolic 5’-nucleotidases CN-II and CN-III can dephosphorylate NMN and NAMN and thus generate NR and NAR in vitro. His-tagged CN-II and CN-III were purified after overexpression in E. coli. Kinetic analyses of their NMN/NAMN 5’-nucleotidase activity were conducted using a colorimetric method to detect inorganic phosphate released during the reaction. The formation of NR and NAR in these reactions was confirmed by HPLC. We conclude that CN-III dephosphorylates both NMN and NAMN, whereas CN-II predominantly dephosphorylates NAMN. Similar to their counterpart from yeast, the human 5’-nucleotidases require millimolar concentrations of NMN or NAMN for efficient catalysis in vitro. Since such concentrations are unlikely in the physiological range, additional cofactors, post-translational modifications or activation signals might be required for efficient riboside generation from NMN and NAMN in vivo. We have also studied the binding of NMN and NAMN to CN-II and CN-III 5’-nucleotidases using molecular docking approach.

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SW02.W10–34

Promoter methylation profiles of inflammatory response genes is affected by stearoyl-CoA desaturase in 3T3 adipocytes

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DNA methylation is a major epigenetic modification that controls gene expression in physiologic and pathologic states. Metabolic diseases such as diabetes and obesity are associated with profound alterations in expression of inflammatory genes. The aim of the present study was to elucidate whether stearoyl-CoA...
desaturase 1 (SCD1), an important control point in lipid metabolism and body weight regulation, affects DNA methylation in adipocytes. Our study showed that the level of global DNA methylation is significantly reduced in 3T3-L1 cells overexpressing SCD1. In contrast, both the silencing of SCD1 gene expression by siRNA and the treatment of cells with specific SCD1 inhibitor resulted in hypermethylation of DNA in 3T3-L1 adipocytes. The changes in the level of DNA methylation correlated with the activity and expression level of methyltransferase 3a (DNMT3a). Interestingly, DNMT1 and DNMT3b were not affected by SCD1 expression. The effect of SCD1 on global DNA methylation was most pronounced in fully differentiated adipocytes. While methylation of adipogenic genes (C/EBPβ, C/EBPδ and PPARγ) was not affected by SCD1, promoter methylation profiles of inflammatory response genes (Il17a, Il10ra, Il6st, Cx3 cl1, Tgfb1, Lib) were related to the SCD1 expression/activity in 3T3 adipocytes. Obtained results show that SCD1 regulates the level of DNA methylation in adipocytes and suggest that the mechanism by which SCD1 affects obesity-related disorders may involve changes in methylation of the inflammatory gene promoters. Support NCN UMO-2011/03/B/NZ4/03055.

**SW02.W10–35**

Stearoyl-CoA desaturation is upregulated in exercise training-induced left ventricular hypertrophy

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The physiological cardiac hypertrophy, caused by endurance training, shows enhancement of cardiac function at rest and during exercise and is not a risk factor for heart failure. Upregulation of several fatty acid oxidation genes was observed in adaptive hypertrophy, however, it remains to be established whether physiological left ventricle hypertrophy affects the expression of genes involved in lipogenesis and in turn affects cardiac lipid content. In the present study we show that physiological hypertrophy was accompanied by an increased expression of sterol regulatory element-binding protein 1c (SREBP-1c), stearoyl-CoA desaturase (SCD)-1, SCD2, glycerol-3-phosphate transferase, acyl-CoA synthase and the protein levels of SREBP-1 and SCD1. SREBP-1 protein level and activity are regulated by Akt signaling. We therefore measured the level of phosphorylation of Akt in myocardium of the experimental animals. The ratio of pAkt/Akt was two fold higher in trained rats compared with sedentary group. Exercise training did not affect cardiac free fatty acid, diacylglycerol, triglyceride, and phospholipid concentrations underlying balanced lipid utilization in the trained heart. Because SCD1 and SREBP-1 were shown to be involved in the regulation of the ability of the heart to respond to stress we hypothesize that increased lipogenesis is one of the step in the development of maladaptive left ventricle hypertrophy.


**SW02.W10–36**

Oxidative and nitrosative stress responsive genes as targets for the search and development of compounds with anti amoebic potential

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The protozoan Entamoeba (E.) histolytica is the etiologic agent of amoebiasis, a global leading cause of death by parasitic infection. The parasite invades and disrupts the host colon, causing severe tissue destruction and inflammation. Upon host infection, E. histolytica is confronted with reactive oxygen and nitrogen species. We have previously identified oxidative and nitrosative stress responsive genes using whole-genome expression profiling.

Under the premise that these stress resistance genes are related to E. histolytica pathogenic potential, we are characterizing from a structural-functional viewpoint a subset of such genes, overexpressing them in bacteria and studying the recombinant proteins by biochemical and biophysical techniques. The purified proteins are also screened for compounds with potential inhibitory effect by differential scanning fluorimetry, a high throughput method that allows assessing the effect of compound binding on the protein's conformational stability. The targets under study comprise proteins of unknown and known function, particularly enzymes involved in the sulfur-containing amino acid metabolism that lack structural homologues in the host and have been validated targets for new anti amoebic drugs.

EHI_056680 encodes a small hypothetical protein herein named H. histolytica stress-induced adhesion factor (EhSIAF). EhSIAF overexpression in E. histolytica enhanced its survival under oxidative stress and resulted in a virulent-related phenotype: increased adherence to healthy and apoptotic mammalian cells, impaired ability to destroy mammalian cell monolayers and decreased motility. EhSIAF was successfully expressed in bacteria and purified to homogeneity. Its biophysical characterization and a search for compounds with binding-inhibitory effect are underway.

Focusing on the validated drug target O-acetylserynine sulhydrylase (EhOASS), we have revisited its kinetic parameters, obtaining a $K_M$ of 5 μM (~100-fold lower than that previously reported), and identified a small set of compounds with promising inhibitory effect on the in vitro enzymatic activity, which are currently under development for further improvement.

The most promising compounds will be tested on living amoebae for cytotoxicity and effect on virulence-related traits.

**SW02.W10–37**

Pre-steady-state kinetics of substrate recognition and processing by Citrobacter freundii methionine γ-lyase

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Methionine γ-lyase (EC 4.4.1.11, MGL) is a pyridoxal 5'-phosphate dependent enzyme found in some bacteria, Archaebae, and primitive protozoa. MGL catalyzes the irreversible γ-elimination of L-methionine to give methanethiol, α-ketobutyrate and ammo-
nia. The enzyme also catalyzes β-elimination reaction of L-cysteine and S-substituted L-cysteines and γ- and β-replacement reactions of L-methionine and L-cysteine and their analogs. The absence of this enzyme in mammals allows to consider it as a biochemical target for a design of new antipathogenic agents. For this purpose, elucidation of MGL mechanism is crucial. Many its features remain poorly explained despite the availability of structural information on some enzyme’s complexes.

In this study, we analyzed the MGL catalytic mechanism of γ-elimination reaction using stopped-flow kinetics with single-wavelength absorbance detection of transient intermediates. The interaction of MGL with L-methionine (Met), L-vinylglycine (Gly(vinyl)), L-methionine sulfone (MetO₂) and O-acety-L-homoserine (Hse-OAc) have been studied using registration of absorbance at 420, 320 and 480 nm. The absorbance time-course curves were different for all substrates. The kinetic scheme was proposed and the intrinsic rate constants of main steps of γ-elimination reaction were determined using a global fitting procedure. The obtained data are important for understanding characteristics of elementary stages of γ-elimination reaction.

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The cys-loop ligand-gated ion channel superfamily includes the nicotinic acetylcholine (nAChR), serotonin 5-HT_3, GABA_A, GABA_C, and glycine receptor channels. These receptor channels are pentameric assemblies, with each subunit arranged around a central ion-conducting pore. The binding of ligand to the extracellular interface between two subunits induces channel opening. With the discovery and crystallization of a molluscan ACh-binding protein, a soluble pentameric protein that is analogous to the extracellular ligand-binding domain of the cys-loop receptors, along with the 4Å resolution of the Torpedo nAChR, much has been learned about the structure of the ligand binding domain and the channel pore, as well as major structural rearrangements that may confer channel opening and function.

The cys-loop receptor channels are widely expressed in the nervous system (both central and peripheral) where they regulate excitability and neurotransmitter release, as well as at the endplate where they induce neuromuscular contraction. Furthermore dysfunctions in these receptors are thought to be involved in a variety of diseases and disorders, including (but not limited to) Alzheimer’s disease, Parkinson’s disease, epilepsy, schizophrenia, and myasthenic syndromes. Presently the cys-loop receptors serve as molecular targets for a variety of clinically important drugs, such as muscle relaxants, tranquillizers, anticonvulsants, and anti-emetics. Thus, these receptors are presently critical targets for the development of therapeutics to treat a variety of neurological diseases and disorders.

I will discuss the advances and challenges in studying the structure and function of the cys-loop receptor channel superfamily. I will start with some lessons learned in the past ten years about Acetylcholine Binding Protein (AChBP) structure and function, and I will focus on how detailed information was generated on ligands interacting with these receptors, with a particular emphasis on compounds acting at the nicotinic acetylcholine- and 5-HT_3 receptors.

**SW03.S11–2**

**Neurotoxic assistants in research on nicotinic receptors**

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Snake venom protein alpha-neurotoxins allowed 40 years ago to isolate the first nicotinic acetylcholine receptor (nAChR) from the Torpedo electric ray. A decade ago alpha-neurotoxin helped to purify the acetylcholine-binding protein (AChBP), an excellent model of the nAChR ligand binding domain. AChBP X-ray analysis paved the way to cryo-electron microscopy structure of Torpedo marmorata nAChR, while crystal structures of the AChBP complexes with agonists and antagonists shed light on the ligand-binding sites in nAChRs. Other sophisticated tools for studies on distinct nAChR subtypes are alpha-conotoxins, neurotoxic peptides from the Conus marine snails. Our earlier work on these two classes of neurotoxins was summarized (Tsetlin et al., Biochem. Pharmacol. 2009), while this talk presents recent results. In N. kouaithia cobra venom, a minor component was identified built of two alpha-cobratoxin molecules connected by two intermolecular disulfides. Such post-translational modification endowed alpha-cobratoxin with a novel property: in addition to blocking muscle-type and neuronal alpha7 nAChRs, it inhibits alpha3beta2 nAChRs. According to X-ray structure, intermolecular disulfides were formed due to disruption and reorganization of the intramolecular disulfides Cys26-Cys30 (Osipov et al., J. Biol. Chem., 2012). Analysis of the of the Acanthoicis feae viper venom gave a 21-membered peptide azemipsin selectively inhibiting muscle-type nAChRs, but containing no disulfides (Utkin et al., J. Biol. Chem., 2012); all earlier known proteins and peptides from animal venoms targeting nAChRs have 1–5 disulfides. A series of alpha-conotoxin PnIA analogs with 1–4 substitutions were designed and synthesized, some possessing higher affinity for AChBP or alpha7 nAChR (Kasheverov et al., Marine Drugs, 2011). The three-finger spatial structure of snake neurotoxins is present in Lynx 1 protein of mammalian brain, membrane-tethered by GPI anchor near nAChRs. Water-soluble domain of Lynx1 lacking GPI anchor (ws-lynx1) was expressed in E.coli. It competed with alpha-bungarotoxin for binding to AChBP and Torpedo nAChR, but not to alpha7 nAChR. However, at 10 μM it inhibited currents in alpha7 nAChR (Lyukmanova et al., J. Biol. Chem., 2011). Thus, at muscle-type nAChRs ws-lynx1 binds at classical site for agonists and competitive antagonists, but outside it at neuronal nAChRs. The difference between the strong inhibitory action of alpha-neurotoxins and modulatory effects of Lynx1 may be due to the nanomolar affinity and irreversibility of binding for the former and micromolar affinity and fast washout for the latter.

**SW03.S11–3**

**P2X and NMDA receptors mediate fast sodium signalling in cortical astroglia**

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Integration and information processing in the brain occurs through close interactions of two cellular circuits, neuronal and glial. Ionotropic receptors mediate neurone-driven signals to astroglial cells in various brain areas including neocortex, hippocampus and cerebellum. Glutamate and ATP are the major neurotransmitters responsible for signalling in neuronal-glial networks. Glial and neuronal NMDA receptors are functionally and structurally different; the glial receptors are weakly sensitive to the extracellular magnesium block, which may indicate a predominant expression of the NR3 receptor subunit. Purinergic neuronal-glial transmission is mediated through P2Y metabotropic and P2X ionotropic purinoreceptors. The P2X_1,2 receptors are ubiquitously expressed in astroglia and their activation trigger intracellular Ca^{2+} signalling. The ionotropic receptors are much more territorially restricted; P2X-mediated responses were hitherto found only in cortical astrocytes. Cortical astrocytes express P2X_1,5 purinoceptors that are characterised by very high sensitivity to ATP (EC_{50} ~ 50 nM) and weak desensitization. In the
cortex, astroglial NMDA and P2X<sub>1</sub> receptors are activated upon physiological synaptic transmission. Spontaneous synaptic currents, mediated by NMDA and P2X<sub>1</sub> receptors were also readily recorded from cortical astrocytes, indicating the close proximity of some areas of glial membranes to the sites of neurotransmitter release from the neuronal terminals. Activation of ionotropic receptors trigger rapid signalling events in astroglia; these events, represented by local Ca<sup>2+</sup> or Na<sup>+</sup> signals provide the mechanism for fast neuronal-glial signalling at the level of individual synapse. In particular spatio-temporally organised [Na<sup>+</sup>] dynamics regulate diverse astroglial homeostatic responses such as metabolic/signalling utilization of lactate and glutamate, transmembrane transport of neurotransmitters and K<sup>+</sup> buffering. Near-membrane [Na<sup>+</sup>] transients determine the rate and the direction of the transmembrane transport of GABA and Ca<sup>2+</sup>.

**SW03.S11–4**

**Transmitter receptors in microglial cells**

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Microglial cells are the immune competent cells of the central nervous system. They are activated in any pathology which triggers proliferation, migration to the site of injury and release of substances such as cytokines. They also have the capacity to sense neuronal activity since they express functional neurotransmitter receptors. We have identified functional receptors for ATP, GABA, dopamine, noradrenaline, serotonin, endothelin and carbachol. Transmitter receptors were first identified in cultured microglia, but are now confirmed to be expressed in situ or in freshly isolated microglial cells. Activation of transmitter receptors controls distinct microglial functions such as phagocytic activity, their ability to move processes to the site of injury, cytokine release and migration. The different transmitters have quite distinct effects. While ATP, for instance, enhances the phagocytic activity, serotonin down regulates it. Moreover, microglial cells are a heterogeneous population with respect to the expression of functional transmitter receptors. Defined receptors such as for endothelin or serotonin are only expressed by a subpopulation of microglia. The population of responding microglia can also change in pathology.

**References**


**SW03.S11–5**

**Role of extracellular purines in the control of stressful brain dysfunction**

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ATP is recognized as an extracellular signaling molecule and has been proposed to act as a danger signal upon brain insults. We now aimed to probe the role of extracellular ATP in different animal models of brain disease. In a rat model of Parkinson’s disease (6-hydroxydopamine), the P2 receptor (P2R) antagonist PPADS (1 nmol, icv) was devoid of effects, whereas the P2X<sub>7</sub>R antagonist brilliant blue G (45 mg/kg, ip) prevented motor deficits through a combined control of synaptotoxicity, neurotoxicity and gliosis. In contrast, in a rat model of status epilepticus triggered by kainate (10 mg/kg), PPADS or the P2Y1R antagonists MRS2179 or MRS2500 (0.5–1 nmol, icv) attenuated both cell death and microgliosis in the hippocampus. Likewise, in a mouse model of brain ischemia (MCAO), PPADS, MRS2179 or MRS2500 (0.5–1.0 nmol, icv) decreased the infarcted area, the MCAO-induced locomotor activity and memory impairment as well as neurotoxicity, but not astrogliosis of microgliosis. P2Y1R blockade (pharmacological or genetic) also prevented memory impairment and synaptotoxicity in a mouse model of Alzheimer’s disease (icv injection of beta-amyloid peptide 1–42, 0.5 nmol). Notably, we found that extracellular ATP was mandatory to enable glutamate to cause neurotoxicity; this seems to be due to a P2Y1R-mediated control of NMDA receptor over-activation, namely in synaptic regions. Finally, we also unravelled the importance of ATP as a source of adenosine. Indeed, the blockade of ecto-5'-nucleotidase blunts the activation of adenosine A2A receptors, controlling synaptic plasticity and sensitization to drugs of abuse. This confirms the key role of extracellular ATP as a danger signal upon noxious brain conditions mainly through P2Y1R-mediated control of NMDA receptor-mediated neuronal damage, and also highlights the interplay between different purinergic systems in the control of stressful brain conditions (Supported by FCT).

**SW03.S11–6**

**An investigation of the mechanism of hydrogen sulphide mediated uterine relaxation**

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Endogenously produced hydrogen sulphide (H<sub>2</sub>S) is recognized as a gaseotransmitter, which appears to be a signaling molecule in rat uterus. However, the signal transduction pathways including ion channels regulated by H<sub>2</sub>S as well as other targets involved in the effects of H<sub>2</sub>S have been unclear and were investigated in the present study.

The effects on contractility in response to sodium sulphide Na<sub>2</sub>S (a hydrogen sulphide donor), were examined on myometrial strips from virgin Wistar rats. Dose-dependent and significant decreases were observed in spontaneous contractions with increasing concentrations of Na<sub>2</sub>S (20 × 10<sup>-6</sup> M–200×10<sup>-6</sup> M). In comparison to spontaneous contractions the relaxant effects of H<sub>2</sub>S on Ca<sup>2+</sup>- activated uteri was with less potency. These effects were underlined with increased activity of GSHPx (glutathione peroxidase). However, an increase in superoxide dismutase activity was found exclusively in Ca<sup>2+</sup>-stimulated uteri.

H<sub>2</sub>S relaxation of uterus seems not to be mediated by K<sub>v</sub> channel since it was not affected by the 4-aminopyridine (1 mM). Pretreatment with glibenclamide (2 × 10<sup>-6</sup> M) (K<sub>A TP</sub> channel inhibitor) slowed down H<sub>2</sub>S induced relaxation. Instead of inhibition, methylene blue (1 × 10<sup>-6</sup> M) (soluble guanylyl cyclase inhibitor) potentiated the H<sub>2</sub>S-induced relaxation of spontaneous contraction. 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS) (100 × 10<sup>-6</sup> M) caused a significant rightward shift of the Na<sub>2</sub>S concentration-response curve, suggesting that H<sub>2</sub>S may target Cl<sup>-</sup> channels or Cl<sup>-</sup>/HCO<sub>3</sub> exchange channel.

H<sub>2</sub>S relaxation of uterus seems to be dependent on the extracellular calcium entry and are DIDS sensitive. Although the biologically relevant chemistries of H<sub>2</sub>S and HS<sup>-</sup> display some similarities, it is clear that they have distinct chemical properties and probably different targets. Recent reports have emphasized Cl<sup>-</sup> channels as potential sulphide targets and transport of HS<sup>-</sup> anions through these channels is under investigation in our laboratory.
During glycerol fermentation at pH 7.5 the H₂ oxidation activity transcriptional activator of Hyd 3 and possibly Hyd-4 [2], into FhlA protein, which is a F₀F₁-activity and/or to H⁺-motive force generation. Especially F₀F₁ were coupled via H₂ oxidation and reverse H⁺ cycling. during glucose fermentation at different pH Hyd enzymes and Hyd enzymes resulted in the increase of the membrane potential. and producing activity, H⁺ transport across the membrane and of Hyd-1 was directly related to F₀F₁, meanwhile deletion of all membrane vesicles F₀F₁-ATPase activity were investigated. It is produce H₂ or to work in a reverse way might form some redox critical one.

Escherichia coli is able to process four [Ni-Fe] hydrogenases (Hyd), three of which are characterized well. The genes of the ‘silent’ fourth Hyd have been revealed but no any proteins have been isolated and characterized in vitro. These Hyd enzymes can work in H₂ oxidizing or producing mode depending on pH and carbon source and are reversible [1]. The activity of different Hyd is suggested to form H₂ recycling pathways and partially to depend on H⁺ transport across the membrane. If one part of these pathways is disrupted Hyd might be switching into the non-active state. In the present study Hyd enzymes H₂ oxidizing and producing activity, H⁺ transport across the membrane and membrane vesicles F₀F₁-ATPase activity were investigated. It is noteworthy to mention that H⁺ cycling through F₀ to Hyd to produce H₂ or to work in a reverse way might form some redox pathway to neutralize fermentation acids and to equilibrate cytoplasmatic pH. It was shown that during single or mixed carbon sources fermentation H₂ oxidation or production are coupled to F₀F₁-activity and/or to H⁺-motive force generation. Especially during glucose fermentation at different pH Hyd enzymes and F₀F₁ were coupled via H₂ oxidation and reverse H⁺ cycling. During glycerol fermentation at pH 7.5 the H₂ oxidation activity of Hyd-1 was directly related to F₀F₁, meanwhile deletion of all Hyd enzymes resulted in the increase of the membrane potential. This might point out Hyd impact into the H⁺ transport. Moreover, during mixed carbon sources (glucose and glycerol) fermentation at acidic pH involvement of FhIA protein, which is a transcriptional activator of Hyd 3 and possibly Hyd-4 [2], into the H₂ cycling and H⁺ transport pathways both is proposed.

References

**SW03.S11–8**

**Escherichia coli** hydrogenases and the F₀F₁-ATPase are coupled via H₂ forming and H⁺ transporting pathways

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**Escherichia coli** is able to process four [Ni-Fe] hydrogenases (Hyd), three of which are characterized well. The genes of the ‘silent’ fourth Hyd have been revealed but no any proteins have been isolated and characterized in vitro. These Hyd enzymes can work in H₂ oxidizing or producing mode depending on pH and carbon source and are reversible [1]. The activity of different Hyd is suggested to form H₂ recycling pathways and partially to depend on H⁺ transport across the membrane. If one part of these pathways is disrupted Hyd might be switching into the non-active state. In the present study Hyd enzymes H₂ oxidizing and producing activity, H⁺ transport across the membrane and membrane vesicles F₀F₁-ATPase activity were investigated. It is noteworthy to mention that H⁺ cycling through F₀ to Hyd to produce H₂ or to work in a reverse way might form some redox pathway to neutralize fermentation acids and to equilibrate cytoplasmatic pH. It was shown that during single or mixed carbon sources fermentation H₂ oxidation or production are coupled to F₀F₁-activity and/or to H⁺-motive force generation. Especially during glucose fermentation at different pH Hyd enzymes and F₀F₁ were coupled via H₂ oxidation and reverse H⁺ cycling. During glycerol fermentation at pH 7.5 the H₂ oxidation activity of Hyd-1 was directly related to F₀F₁, meanwhile deletion of all Hyd enzymes resulted in the increase of the membrane potential. This might point out Hyd impact into the H⁺ transport. Moreover, during mixed carbon sources (glucose and glycerol) fermentation at acidic pH involvement of FhIA protein, which is a transcriptional activator of Hyd 3 and possibly Hyd-4 [2], into the H₂ cycling and H⁺ transport pathways both is proposed.

References

**SW03.S11–9**

Adrenergic-like effect of 2,3-dehydrosilybin on perfused adult rat heart

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**Aims:** 2,3-Dehydrosilybin (DHSB), a flavonolignan component of *Silybum marianum*, and an oxidized derivative of silybin (SB), belongs to a class of substances with demonstrated antioxidant properties. DHSB is a candidate chemoprotective agent owing to its potent antioxidant capacity as compared to that of SB and displays cytoprotective properties that are linked to its antioxidant effects. We described recently the interaction of DHSB with cardiomyocyte mitochondria and showed that DHSB attenuated cardiomyocyte damage following hypoxia/reoxygenation by modulating mitochondrial bioenergetics. The aim of the current study was to examine the effect of DHSB on isolated perfused rat heart.

**Methods and Results:** Rats, randomly divided into four groups (*n* = 3 in each group), were anesthetized (2% Rometar 0.5 ml + 1% Narkamon 10 ml, dose 0.5 ml solution/100 g body weight) and heparinized (500 IU, i.p.) before the experiments. Hearts were excised, placed in ice-cold perfusion buffer and perfused according to Langendorff at 37°C. Control hearts were perfused for 50 min with Krebs-Henseleit buffer (K-H buffer; 118 mM NaCl, 5.9 mM KC1, 1.75 mM CaCl₂, 1.2 mM MgSO₄, 0.5 mM EDTA, 25 mM NaHCO₃, 11 mM glucose) at pH 7.4. The perfusate was gassed with 95% O₂ – 5% CO₂. Treated hearts were perfused for 25 min with Krebs-Henseleit buffer and than with buffer containing 10 µM DHSB or 10 µM quercetin or 100 nM bradykinin for 15 min, followed by 10 min of washout. The effects of all substances tested on functional parameters during the experiment were compared with a control group. Left ventricular developed pressure (LVPD), end-diastolic pressure (LVEDP) and contractility (dP/dt max) were measured using a water-filled latex balloon, which was placed in the left ventricular chamber and connected to an analog converter.

Control hearts and hearts treated with quercetin demonstrated no significant changes throughout the perfusion period. Bradykinin caused a vasodilator effect as expected. DHSB caused prompt and dynamic elevation of blood pressure, increase of heart rate and subsequent elevated blood pressure. Blood pressure and contractility were reduced to initial values and heart rate was decreased to half value during washout period by K-H buffer. The functional changes in heart output in the presence of DHSB were accompanied by morphological changes of the heart: enlargement of the organ and uneven surface of ventricle.

**Conclusion:** DHSB causes positive ino-, dromo-, chrono and bathmotropic changes which resemble catecholamine stimulation. However, it is not clear whether DHSB acts through adrenergic receptors because such an oxygen wasting effect in the whole organ is consistent with our previously published uncoupler-like behavior of DHSB in isolated mitochondria.

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Three-dimensional structure of human Kv10.2 ion channel suggests mechanism for its activation

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Voltage-gated potassium K⁺ channels are widely distributed in the central nervous system, endocrine cells, heart, lung, liver. The members of the Kₐ10-12 subfamilies are characterized by extremely long N- and C-terminal intracellular tails, which possess a number of structural domains. The N-terminal PAS domain plays an important role in activation (Wray, 2004), and is thought to alter the rate of deactivation, possibly by binding at or near the S4-S5 linker at the inner mouth of the pore (Cabral et al., 1998, Wang et al., 1998). Here we present two 3D structures of the full-length and truncated human Kᵥ10.2 channels, obtained by single particle EM. For interpretation of the 3D structures we used homology modeling. We demonstrate that the full length Kᵥ10.2 channel is build according to the ‘hanging gondola’ type (Sokolova et al., 2001), and its cytoplasmic and transmembrane parts are connected by linkers. The cytoplasmic part includes the interconnecting PAS and cNBD domains. Unlike in the related HCN (Locatelli et al., 2011) and MloT1K1 (Chen et al., 2007) channels, in the full-length Kᵥ10.2 channel the C-terminal cNBD domains do not form a tetramer. Deletion of the PAS domain leads to the conformational change in the cytoplasmic part of the channel, resulting in formation of the tetramer of the cNBD domains. Thus we have for the first time demonstrated that the cell-attached patch clamp technique had conductances of 1.2, 4.5, and 17 pS (I_max, I_m, and I_min respectively), but it remained unknown which of these channels were composed of TRPC1 proteins. To resolve this issue, we performed single-channel analysis in HEK293 cells transfected with siRNA against TRPC1 or a plasmid coding for TRPC1.

The involvement of TRPC1 in the SOC influx was initially evaluated using the Ca²⁺ imaging method based on Fura-2 fluorescence. TRPC1 suppression in HEK293 cells appeared to result in significant reduction of the calcium influx evoked by depletion of intracellular calcium stores by Tg. To verify this result, we performed a series of whole-cell current recordings. The amplitude of Tg-induced SOC current in TRPC1-knockdown cells proved to be approximately 80% lower than in scrambled siRNA-treated cells. All these data provide the evidence that TRPC1 proteins are a component of native SOC in HEK293 cells.

Next, we performed single-channel analysis to evaluate the effects of TRPC1 suppression on other store-operated calcium (SOC) channels in HEK293 cells. Calcium channels detected by the cell-attached patch clamp technique had conductances of 1.2, 4.5, and 17 pS (I_min, I_m, and I_max respectively), but it remained unknown which of these channels were composed of TRPC1 proteins. To resolve this issue, we performed single-channel analysis in HEK293 cells transfected with siRNA against TRPC1 or a plasmid coding for TRPC1.

Extracellular application of UTP or Tg to siTRPC1-transfected cells resulted in activation of inward currents, and current recording analysis revealed two types of cation channels: I_min and I_m. In control experiments with HEK293 cells transfected with a plasmid coding for nontargeted siRNA, we observed the activity of not only I_min and I_m, but also of I_max channels. The results of single-channel analysis showed that TRPC1 knockdown in HEK293 cells resulted in the disappearance of I_max channels, while the properties of I_min and I_m channels were unaffected.

Experiments described above suggest that TRPC1 forms I_max channels. To verify this hypothesis, we employed the strategy of mouse TRPC1 overexpression in HEK293 cells. Application of UTP or Tg to cell-attached patches of TRPC1-transfected cells activated the channels with properties absolutely identical to those of native I_max channels.

All these data allow us to conclude that TRPC1 protein forms native store-operated I_max channels but is not an essential subunit for other store-operated channel types in HEK293 cells.
SW03.S11–13
Role of dipole potential in the channel-forming activity of cecropin A in planar lipid bilayers
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The effect of dipole modifying agents, flavonoids (phloretin and myricetin) and styrylpyridinium dyes (RH421 and RH237), on the channel forming activity of cecropin A (CA), an antimicrobial peptide from Hyalogloea cecropia, was studied. CA added on the one side of a planar bilayer formed from equimolar mixture of dioleoylphosphoscerine and dioleoylphosphoethanolamine in 0.1 M KCl (pH 7.4) led to the formation of ion channels of different conducance varied from picosiemens to nanosiemens. A mixture of anion-selective, non-selective, and cation-selective species were found. Addition to the bilayer bathing solution of 20 μM phloretin, which decrease the membrane dipole potential (ΔVd = –90 ± 10 mV), led to 4–33-fold reduction of steady-state CA-induced transmembrane current (I) and two-fold decrease of mean single channel conducance (G). The introduction of 20 μM myricetin, which did not practically decrease the membrane dipole potential (ΔVd = –20 ± 5 mV), did not affect I and G. At the same time, increasing the membrane dipole potential due to the introduction of 5 μM RH421 (ΔVd = 60 ± 10 mV) or 5 μM RH237 (ΔVd = 100 ± 5 mV) led to 3–20-fold increase in I and four-fold reduction of G. The observed changes in the channel-forming activity of CA might be attributed to participation of dipole-dipole and charge-dipole interactions in the insertion and aggregation of cecropin molecules in the membrane during the channel formation.

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SW03.S11–14
Electrophysiological properties of native store-operated channels regulated by Stim2 calcium sensors
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In our previous studies on HEK293 and A431 cells we have shown that there are three types of Ca2+ channels in cell-attached patches: I_{max, INS} and I_{max, Imin}. INS and Imin were activated by store depletion, while Imin channels were store-operated in A431 and store-independent in HEK293. We supposed that this difference in regulation could be due to Stim proteins which are the essential regulators of store-operated entry. Current study focuses on the role of Stim1 and Stim2 proteins in regulation of calcium channels.

In HEK293 cells with Stim 1 knockdown thapsigargin (Tg) induced a smaller Ca2+ entry than in control cells. Single-channel data on HEK293 treated with siRNA against Stim1 demonstrated that Tg-evoked activation of I_{max} or INS channels declined, while Imin channels became sensitive to store depletion with 1 μM Tg. It was a surprising result because it is assumed that all store-operated channels are activated by Stim1.

We proposed that Imin might be regulated by some other sensors, possibly by Stim2. In HEK293 cells overexpressing Stim2 Imin channels became store-operated like it was in HEK293 cells with Stim1 knockdown.

We hypothesized that Imin store-operated activity depends on the ratio between active Stim2 and Stim1 proteins. There is a big difference in calcium sensitivity between Stim1 and Stim2 proteins. Stim1 proteins are known to be activated only by strong Ca2+-store depletion, which can be evoked by micromolar concentration of thapsigargin. While Stim2 proteins are stimulated even by small changes in Ca2+ concentration in the store, which did not activate Stim1. Such partial store depletion could be evoked by 10 nM thapsigargin. Addition of 10 nM thapsigargin to HEK 293 cells activated calcium currents through Imin channels.

Therefore we might conclude that in native cells Stim2 is capable of activating Imin channels. We speculate that difference in Stim1 to Stim2 ratio might account for Imin channels being store-operated in A431 cells and store-independent in HEK293.

SW03.S11–15
The role of PSD-95 in the rearrangement of Kv1.3 channels to the immunological synapse
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T lymphocytes play a crucial role in the adaptive immune response. They are activated through an interaction with antigen presenting cells during the establishment of the immunological synapse (IS). It is well documented that several proteins accumulate in the IS. We have previously shown that one of these molecules is the voltage-gated potassium channel, Kv1.3. It is well known, that PSD-95 and SAP97 are adaptor proteins that regulate the polarized cell surface expression and localization of different Kv1 channels in neurons. We investigated whether these proteins are expressed in non-excitable human T cells and affect the redistribution of Kv1.3 channels into the IS.

We found that PSD-95 and SAP97 are expressed in Jurkat cells and interact with the C terminus of Kv1.3. The interaction between PSD-95 or SAP97 and Kv1.3 in Jurkat was disrupted either by the expression of a C-terminal truncated Kv1.3, which lacks the binding domain for these proteins, or the expression of PSD-95 or SAP97 was knocked down using specific siRNA. The recruitment of Kv1.3 into the IS was inhibited in the truncated channel expressing and the PSD-95 knockdown cells: the fraction of cells showing polarized Kv1.3 expression upon engagement in an IS was significantly lower than in control cells expressing the full length Kv1.3, and the rearrangement of Kv1.3 did not show time dependence. However, Jurkat cells expressing the full length channel showed marked time-dependence in the recruitment into the IS peaking at 1 min after the conjugation of the cells. These results demonstrate that PSD-95 participates in the targeting of Kv1.3 into the IS, implying its important role in human T cell activation.

SW03.S11–16
The pathological pathway of endoplasmic reticulum calcium overload connected with Familial Alzheimer’s disease
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Familial Alzheimer’s disease (FAD) is caused by mutations in presenilin-1 (PS1) gene in approximately 50% of cases. The M146V mutation in PS1 gene was shown to cause calcium homeostasis impairments independently on PS1 catalytic activity
as the gamma-secretase enzyme subunit. It was found that M146V mutant increases endoplasmic reticulum (ER) calcium content in neurons. To determine molecular players involved in the calcium overload in ER we performed calcium imaging experiments with Fura2-AM with human neuroblastoma SK-N-SH cell line, transfected with PS1-M146V mutant or PS1-WT as a control. An ionomycin-sensitive pool was used to access the level of calcium concentrations in internal stores including ER. PS1-M146V expression increased the calcium concentration level in internal stores of SK-N-SH cells comparing to control cells with PS1-WT. Suppression of ER calcium sensor STIM2 expression by shRNA in cells with PS1-M146V mutant reduced the Ca2+ concentration to the control value. As long as STIM2 can activate store-operated calcium (SOC) channels formed by TRPC1 subunit, we assumed that concentration of Ca2+ in ER increased due to activity of SOC channels and excessive calcium entry. Suppression TRPC1 expression by shRNA retrieved the calcium concentration level to control values. In addition PS1-M146V mutant expression reduced the total protein level of TRPC1 in SK-N-SH cells. We suppose that the decrease of TRPC1 expression is a cell compensatory response to high activity of STIM2 in PS1-M146V mutant expressing cells. Therefore the pathological response is a cell compensatory response to high activity of STIM2 in SK-N-SH cells. To determinate molecular players involved in the pathological pathway of ER overload connected with FAD PS1-M146V mutant includes TRPC1 SOC channels activation by calcium sensor STIM2.

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**SW03.S11–18**

Abscisic acid transport in human erythrocytes

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Abscisic acid (ABA) is a plant hormone involved in the response to abiotic stress and the control of seed germination. Recently, ABA has been demonstrated to be present and active also in mammals, where it stimulates the functional activity of innate immune cells and insulin release.

The ABA receptor in mammalian cells, LANCL2, is located at the intracellular side of the plasmamembrane, where it activates a trimeric G-protein upon ABA binding. The fact that LANCL2 is not a transmembrane protein requires ABA to cross the plasmamembrane to bind to its receptor.

Here we investigated the mechanism underlying ABA transport across the plasmamembrane of human erythrocytes.

Influx and efflux of [H3]-ABA occurs across intact erythrocytes, as detected both by radiometric and chromatographic methods, as well as across resealed erythrocyte ghosts, but not across liposomes. ABA binds specifically to band 3 (the erythrocyte anion transporter), as detected with biotinylated ABA by Western blot on erythrocyte membranes.

Finally, proteoliposomes reconstituted with human purified band 3 transport ABA.

These results identify band 3 as an ABA transporter in human erythrocytes and open the way to an investigation into the role of this protein in the physiology of ABA in mammals.

**SW03.S11–18**

Infertile HSL-knockout mouse testis shows class B scavenger receptor up-regulation, disrupted lipid raft microdomains, and activated p-ERK, p-AKT, and p-SRC

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There is a tight relationship between fertility and changes in cholesterol metabolism during spermatogenesis. In the testis, class B scavenger receptors (SR-B) (SR-BI, SR-BII, and LIMP II) mediate the selective uptake of cholesterol esters from HDL, which are hydrolyzed to unesterified cholesterol by hormone sensitive lipase (HSL). HSL is critical because HSL knockout (KO) male mice are sterile.

**Objective:** To determine the effects of the lack of HSL in testis on the expression of SR-Bs, lipid raft composition, and related cell signalling pathways.

**Methods:** WT and HSL-KO mice testis were frozen or embedded in paraffin to study protein expression and immunolocalization. Seminal fluid was obtained from epididymis for spermatoozoa counts and motility. Mice testis lipid rafts were isolated by sodium carbonate extraction and sucrose gradient fractionation.

**Results:** HSL-KO mouse testis presented altered spermatogenesis associated with decreased sperm counts, sperm motility and infertility. In WT testis, HSL is expressed in elongated spermatids, SR-BI in Leydig cells and spermatids, SR-BII in spermatocytes and spermatids but not in Leydig cells, and LIMP II is present in Sertoli and Leydig cells. The lack of HSL in mouse testis induces augmented expression of SR-BI, SR-BII, and LIMP II, alters caveolin-1 localization in lipid raft plasma membrane, and induces activation of key proteins (p-ERK, p-AKT, and p-SRC) in cell signaling pathways involved in differentiation, proliferation, and other cellular processes during spermatogenesis.

**Conclusions:** The lack of HSL dramatically disrupts cholesterol homeostasis in the testis, with augmented expression of SR-Bs (SR-BI, SR-BII, and LIMP II), altered lipid raft microdomains, and activation of different signaling pathways with relevance in spermatogenesis.

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**SW03.S11–19**

An outwardly rectifying chloride current of *Xenopus tropicalis* oocytes

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Oocytes of *Xenopus* present an outward rectifying current that has two components: (i) one voltage activated and independent of intracellular or extracellular Ca2+, and (ii) a smaller component that is Ca2+ dependent. The properties of the Ca2+-independent current, such as voltage dependence and outward rectification, resemble those of CIC anion channels/transporters. This current is sensitive to NPPB and NFA, insensitive to 9AC and DIDS, and showed a whole-cell conductance sequence of SCN− >I− >Br− >Cl−. RT-PCR revealed the expression in oo-
cytes of CIC-2 to CIC-7, and major reductions of current amplitudes were observed when a CIC-5 antisense oligonucleotide was injected into oocytes. The $\text{Ca}^{2+}$-dependent component was abolished after injection of BAPTA or EGTA, whereas $\text{Mg}^{2+}$ inhibited the current. This component was blocked by 9-AC, NFA, and NPPB, whereas DIDDS did not elicit any evident effect. The ion sequence selectivity was $\text{SCN} = \Gamma > \text{Br}^{-} > \text{Cl}^{-}$. To try to determine the molecular identity that gives rise to this component we assessed by RT-PCR the expression of the $\text{Ca}^{2+}$-dependent Cl$^{-}$ channel TMEM16A, which was found to be present in the oocytes. However, injection of antisense TMEM16A oligonucleotides did not inhibit the transient outward current. This result fits well with the electrophysiological data, since the observed currents do not correspond to those of either TMEM16A or Bestrophins. Together, these results suggest that CIC-5 is a major, but not the sole channel responsible for this outwardly rectifying Cl$^{-}$ current.

**SW03.S11–20** Contribution of transmembrane residues to sensitization and pore dilation of the rat P2X7 receptor

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P2X receptors (P2XRs) are membrane cation-permeable channels that open in response to the binding of extracellular adenine 5'-triphosphate (ATP). In mammals, seven P2X subunits (P2X1–7) have been identified which form functional trimeric homomers or heteromers. The kinetics of P2X7R widely differs from other subtypes. The most striking feature is that during sustained agonist application the receptor does not desensitize as other P2X subtypes, but its pore dilates reaching the permeability for organic cations. Formation of a pore in the plasma membrane is associated with uncontrolled $\text{Ca}^{2+}$ influx and receptor sensitization. The aim of this work was to study the role of several conserved residues (Y40, G345, D352) and two non-conserved residues (G338, L341) in both transmembrane domains (TM1, TM2) in kinetics of the P2X7R. Identical residues have been shown previously to be crucial for P2X4R function (Jelinkova et al., 2008). Pairwise identity of P2X subunits in amino acid sequences is 39–55%; and the P2X4 and P2X7 subunits are the most closely related pairs. The selected residues were substituted to alanine, single-point mutants expressed in HEK293, and agonist-induced currents were measured by whole cell patch clamp recording technique. We tested maximum current amplitude and sensitivity to BzATP, a selective P2X7 agonist, and in some mutants also to other agonists. Brief (2–10s) or long (60s) applications of BzATP were used to estimate EC$_{50}$ values for receptor in normal and diluted (sensitized) state, respectively. The membrane expression of low-responsive mutants was examined by western blotting. Our results showed that substitution of conserved TM1 tyrosine abolished the ability of the P2X7 to dilate the pore and to increase the sensitivity to agonists. Substitution of Gly338 in the TM2 reduced receptor expression in the plasma membrane, but residual receptors in the membrane exhibited significantly increased pore conductance and high sensitivity to all agonists. Dilation and sensitivity was also affected by substitution of L341 and G345. The D352A mutant was not functional. These results showed a correlation between P2X4 and P2X7 receptors and identified several TM residues that play important role in channel gating and sensitivity of the P2X7 receptor.

**SW03.S11–21** Purinergic signalling engage both intracellular $\text{Ca}^{2+}$ mobilization and extracellular $\text{Ca}^{2+}$ inflow to regulate human megakaryocyte motility and platelet production

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During differentiation megakaryocytes (Mks) migrate from the bone marrow osteoblastic niche to the vascular niche, where they convert the bulk of their cytoplasm into multiple long processes called proplatelets which protrude into the sinusoid lumen, where platelets are finally released into bloodstream. Platelet production is regulated by a concert of environmental and autocrine factors that coordinate the different steps of megakaryocyte development. We previously showed that Mks express the purinergic receptor P2Y$\text{13}$ and that its interaction with constitutively released adenosine diphosphate (ADP) leads to platelet production. ADP-dependent platelet activation relies on the increase in cytosolic $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]), accomplished by store-operated $\text{Ca}^{2+}$ entry (SOCE), while whether or not this mechanism plays a role in platelet release is unknown. SOCE has been described to be required in different human cell types to promote interaction with extracellular matrix components. Thus, we aimed to investigate the role of ADP-induced SOCE activation in regulating Mk behavior within extracellular matrix environment.

We demonstrated that ADP binding to P2Y$\text{13}$ elicits a rapid increase in [Ca$^{2+}$], followed by a plateau, which is lowered in Ca$^{2+}$-free solution, thereby suggesting the involvement of store-operated Ca$^{2+}$ entry. Therefore, we provided the first evidence that Mks express the major candidates to mediate store-operated Ca$^{2+}$ entry, STIM1 and Orai1, which were functionally activated upon depletion of the intracellular Ca$^{2+}$ pool. The mechanism was impaired by inhibition of phospholipase C, inositol-3-phosphate receptors, or specific store-operated Ca$^{2+}$ channels. Finally, studies on Mks function revealed that Ca$^{2+}$ entry form extracellular media is primarily involved in the regulation of cytoskeleton rearrangement responsible for cell adhesion and motility on extracellular matrix components. Conversely, only Ca$^{2+}$ mobilization form intracellular stores is required to activate signaling cascades that trigger proplatelet formation.

These findings provide the first evidence that ADP engage store-operated Ca$^{2+}$ entry to regulate human Mks behavior and open new perspectives in the evaluation of the signals that control platelet production.

**SW03.S11–22** Neuroprotective efficiency of Sinestrol after bilateral ovariectomy

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Bilateral ovariectomy causes neurodegeneration in the nervous system of rodents and imitates systemic disorders in postmenopausal women’s organism. Synaptic modulation by estrogen is essential to understand the molecular mechanisms of estrogen replacement therapy.
The aim of present work was to determine the action of Sinetrol on rats’ hippocampal synaptic transmission, plasticity and cell survival in condition of bilateral ovarioectomy. Electrophysiological and morpho-histochemical (by revealing of Ca2+-dependent acid phosphatase) studies by extracellular recording of hippocampal single-neuronal spike activity under high-frequency stimulation of entorhinal cortex were performed on: (i) intact Albino rats, (ii) after 8 weeks of ovarioectomy (placebo-control), (iii) after 8 weeks of ovarioectomy (after 3 weeks i/m injection of Sinetrol- 0.1 ml 2%). Our data suggest that ovarioectomy reduces hippocampal synaptic activity and failure the balance of excitatory and inhibitory responses of norm. After 8 weeks following ovarioectomy in hippocampal neurons dominate effects of tetanic depression in combination with posttetanitic potentiation (45%) and areactive neurons (21%).

Thus Sinetrol promote the reorganization of neuronal circuitries of cortex-hippocampus by modulation of anomalous synaptic activity, as well as the balance of areactive and reactive units. Morpho-histochemical analysis of studies by revealing Ca2+-dependent acid phosphatase testifies that Sinetrol enhances phosphorilation processes providing optimization of regeneration process following ovarioectomy.

SW03.S11–23
Influence of residues in low-conserved regions near the ATP-binding site of P2X4 receptor on channel gating

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Members of family of purinergic P2X receptors are widely expressed in vertebrates as well as in invertebrates. They play role in many physiological and pathophysiological processes, the P2X4 receptors are involved in sensations of inflammatory pain, release of interleukins or long term potentiation, for example. Electrophysiology, crystallography and other studies performed so far provided a lot of information which helped to understand the structure-function relationships of conserved parts of these receptors, however, unstructured low-conserved parts still keep their secrets. The aim of this study was to investigate the role of two non-structuredal domains, D280-Y292 (termed ‘left flipper’) and R203-Y213 (‘dorsal fin’) located nearby ATP-binding site, in the function of the rat P2X4 receptor. These two relatively long loops form interface between subunits and are adjacent to β-sheet and α-helix structures that contain two ATP-binding residues confirmed by crystallization (N293 and L214). We substituted each of these amino acids with alanine and measured agonist concentration producing 50% of effect (EC50) and maximum amplitude of ATP-induced currents from HEK293 cells transfected with wild-type or mutated receptors. We examined also desensitization properties of mutated receptors. Ivermectin, a specific positive allosteric modulator of P2X4 receptor, was used as a tool to estimate the effect of the mutation on receptor-channel binding/gating domain. These experiments showed that alanine substitution of three of eleven residues in the dorsal fin (R203A, N204A and I205A) and four of fourteen residues in the left flipper (D280, R282, N287 and P290) response to ATP with significantly reduced amplitude of maximum current. Most of these receptors and several other mutants (H286A, G291A, L206A and T210A) showed reduced sensitivity to ATP. Contrary to ATP-binding mutants, which were also measured, the amplitude of maximum current in low-functional receptors could be fully restored by the treatment with ivermectin. Any of examined mutants showed significant changes in desensitization kinetics. These results indicate that the non-structuralized segments D280-Y292 and R203-Y213 nearby ATP-binding site are important for channel gating or signal transduction.

SW03.S11–24
Functional expression and axonal transport of alpha7 nAChRs by CGRP-ergic neurons of adult rat dorsal root ganglion

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In recent studies on animal pain models, α7 nicotinic acetylcholine receptor (nAChR) agonists demonstrated analgesic, anti-hyperalgesic and anti-inflammatory effects, apparently acting through some peripheral receptors. Assuming that α7 nAChRs on nociceptive sensory neurons might be responsible for these effects, we investigated the α7 nAChR-expressing subpopulation of dorsal root ganglion (DRG) neurons, specifically, their morphological features, sensory modality and ability to transport α7 nAChR axonally. In addition, the receptor activity and its putative role in pain signal neurotransmitter release were studied. Medium-diameter α7 nAChR-expressing neurons prevailed, although all cell sizes among them were also present. These cells accounted for one-fifth of total medium and large DRG neurons and <5% of small ones. Eighty-three percent of αβ nAChR-expressing DRG neurons were peptidergic nociceptors (CGRP-immunopositive), one half of which had non-myelinated C-fibers and the other had myelinated Aδ- and likely Aβ-fibers, and 15% were non-peptidergic C-fiber nociceptors binding isoleucitin B4. All non-peptidergic and a third part of peptidergic (C- and Aδ-fiber) α7 nAChR-bearing nociceptors expressed vanilloid receptor 1, capsaicin-sensitive heat and mechanical noxious stimulus transducer. Nerve crush experiments demonstrated that CGRP-ergic DRG nociceptors axonally transported α7 nAChRs both to the spinal cord and periphery. α7 nAChRs were functional as their specific agonist PNU282978 evoked calcium rise in DRG neurons enhanced by α7-selective positive allosteric modulator PNU120596, while most of them were C- and Aδ-fiber nociceptors being capsaicin-sensitive. Functional α7 nAChRs are expressed and axonally transported by nociceptive DRG neurons, mainly by medium-sized CGRP-ergic. However, they do not modulate neurotransmitter CGRP and glutamate release from DRG neurons since nicotinic ligands affected neither their basal nor provoked levels, showing the necessity of further studies of the α7 nAChR function.

SW03.S11–25
Plant flavonoids affect membrane activity of antimicrobial agents

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Flavonoids are plant polyphenols imparting flavor and color to fruits and vegetables. Flavonoids are known as powerful antioxidant agents and cell metabolism regulators. Adsorption of flavonoids on the membrane may be accompanied by changes in the properties of the membrane, in particular, its dipole potential, the potential drop on the water-membrane interface. The effects of flavonoids on the channel forming activity of syringomycin E and syringopeptin 22A, the antifungal cyclic lipopeptides from Pseudomonas syringae, surfactin, a lipopeptide antibiotic from Bacillus subtilis, antifungal macrolide polyenes from Streptomyces,
amphotericin B, nystatin and filipin; and antibacterial peptides cecropins from *Hyalophora cecropia* in the planar lipid bilayers were studied. It was found that chalcone phloretin enhances the channel forming activity of lipopeptides *Pseudomonas syringae* and macrocide polyene antibiotics while it leads to decrease of the membrane activity of surfactin and cecropins. Flavonol quercetin increases the activity of filipin while it reduces the activity of amphotericin B. The analysis of the obtained results allowed us to ascertain the main mechanisms of action of flavonoids on membrane activity of antimicrobial agents: the reduction of the membrane dipole potential and direct interaction with the channel forming molecules.

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**SW03.S11–26**

**Single channel recordings of STIM2–operated (Imin) calcium channels in HEK293 cells**

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Activation of phospholipase C mediated signaling pathways lead to calcium release from endoplasmic reticulum (ER) calcium stores. Ca\(^{2+}\) depletion in the ER lumen causes Ca\(^{2+}\) -binding proteins STIM oligomerization into puncta and subsequent activation of plasma membrane calcium channels. These channels are store-operated channels. It is the main pathway of Ca\(^{2+}\) influx in non-excitable cells. There are two homologous of STIM proteins in mammalian cells: STIM1 and STIM2.

Previously we have found \(I_{\text{MSI}}\) channels in A431 and HEK293 cell lines. They can be activated by extracellular addition of UTP or by application of inositol 1,4,5-trisphosphate to internal surface of plasma membrane. \(I_{\text{MSI}}\) channels are activated by passive store depletion in A431 but not in HEK293 cell line. STIM1 knockdown resulted in the reduction of store-operated Ca\(^{2+}\) entry in HEK293 cell line. However \(I_{\text{MSI}}\) channels became store-operated in such cells. Therefore these channels are not regulated by STIM1 and it is suggested that they can be activated by another Ca\(^{2+}\) sensor that is STIM2.

To separate STIM1 and STIM2 activation and amplify the effect of STIM2 we overexpressed STIM2 protein in HEK293 cells. Also we evoked partial depletion of the calcium stores which activates STIM2 but not STIM1 proteins.

By using calcium imaging with fura 2 AM probe we found out that application of 10 nM thapsigargin resulted in partial store depletion in A431 and HEK293 cells because the subsequent addition of 1 \(\mu\)M thapsigargin led to second Ca\(^{2+}\) release. By using patch clamp technique in cell-attached configuration we demonstrated that 10 nM thapsigargin evoked \(I_{\text{MSI}}\) channels activation in HEK293 cells with STIM2 overexpression.

All these data allow us to conclude that STIM2 proteins participate in regulation of \(I_{\text{MSI}}\) calcium channels activity.

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**SW03.S11–27**

**The first single-channel recordings of voltage–dependent ionic channels in dinoflagellates**

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Dinoflagellates represent one of the most successful groups of unicellular marine eukaryotes, which play a crucial role in aquatic ecosystems. These organisms are able to produce potent neurotoxins, such as saxitoxin, gonioautoxin, brevetoxin. Accumulation of neurotoxins in water during harmful dinoflagellate blooms leads to poisoning and death in marine animals and even humans. On the other hand, these toxins are widely used in the pharmaceutical industry as activators and blockers of ionic channels and enzymes, which are required in therapy of various brain and heart diseases. However, ionic channels of dinoflagellates themselves remain almost uninvestigated. The knowledge on their structure and functioning will clarify evolution of ionic channels among eukaryotes and specify physiological features of dinoflagellates. The latter is essential for control and regulation of harmful algal blooms as well as for production of drugs. In our research, we focused on the voltage-gated ionic channels in potentially toxic cosmopolitan dinoflagellates *Prorocentrum minimum*. The most effective method for *in situ* investigation of ionic channels is the patch-clamp technique. Whereas this electrophysiological approach can be easily applied to mammalian cells, it is not the case with protistan cells, which are often motile and have a complex and rigid cell covering. Due to this fact, there is still a little number of the patch-clamp based studies on protists. Dinoflagellates *P. minimum* possess a very complex cell covering consisting of three membrane layers armored with cellulose thecal plates. Therefore, the intact cells are not suitable for the patch formation. We developed the protocol for preparation of *P. minimum* spheroplasts based on cellulose synthesis inhibition. Spheroplasts obtained by this approach are applicable for the gigaseal formation (1–6 GΩm). Furthermore, we managed to get the single channel recordings of voltage–dependent sodium channels. To our knowledge, these are the first patch-clamp recordings of ionic channels in dinoflagellates. The channels demonstrate a positive (ca. +20 mV) reversal potential and high conductivity. Supported by the Russian Foundation for Basic Research grants 12-04-31952, 13-04-00703.

**SW03.S11–28**

**The subunit composition of NMDA receptors in human T-lymphocytes**

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Among ionotropic glutamate receptors, the NMDA subtype is the best studied because of its crucial importance in neuronal function, both in healthy individuals and in pathology. Structurally, the NMDA receptors are a tetramers composed of NR1 subunit and one or more of the NR2 subunits (NR2A–NR2D), which form an ion channel that is highly permeable for K\(^{+}\), Na\(^{+}\), and Ca\(^{2+}\). They may also comprise NR3 subunits. The NR1 subunit is ubiquitously expressed in the CNS. In contrast, the expression of the NR2 subunits is spatially and temporally regulated. In addition to the existence of glutamate receptors in neurons, it has become clear during the last decade that a T-lymphocytes also contain functional NMDA receptors.
The aims of our study were to determine the subunit composition of NMDA receptors in human T-lymphocytes, and to clarify if there were any receptor composition changes during cell activation.

In the first series of experiments we determined the expression of the NMDA receptor subunit mRNA in human T-lymphocytes with RT-PCR analysis. According our data, NR1 subunit mRNA was expressed both in resting and TCR-activated T-lymphocytes, whereas NR2A, NR2C, NR2D transcripts were found mainly in resting cells; the transcripts for NR2B subunit were detected at very low level. In addition, NR3A and NR3B transcripts were also identified both in resting and activated cells. Our data suggest, that TCR stimulation significantly increases the expression of genes encoding for NR1, NR2C, NR2D and NR3A subunits. To examine NR1 subunit protein expression we used specific mAb against an extracellular epitope of the NR1 protein. With flow cytometry and confocal microscopy we clearly demonstrated both surface and intracellular protein expression of NR1 subunits.

Besides, TCR activation of T-lymphocytes results to a significant increase of representation and clustering of NR1 subunit on plasma membrane.

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SW03.S11–29
The role of alpha2 isoform of Na,K-ATPase in the wall of small arteries: contraction, relaxation and intercellular communication
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The α2 isoform of the Na⁺, K⁺-pump (α2 Na⁺-pump) forms in vascular smooth muscle cells (VSMCs) a spatially restricted microdomain with the Na⁺,Ca²⁺-exchanger (NCX). The α2 Na⁺-pump has also been suggested to interact with Src-kinase. Both types of signaling can be involved in the key vascular functions, such as contraction, endothelium-dependent relaxation and VSMC synchronization.

Using in vivo siRNA transfections of rat mesenteric small arteries we have studied the pathways involved in different vascular responses. Small arteries were studied in vitro in isometric myograph. Intercellular communications were evaluated electrophysiologically, using capacitance measurements. [Ca²⁺]o, events were studied with fluorescent and confocal microscopy. Src-kinase signaling was inhibited by genistein and PP2. A membrane-permeable peptide, NaKtide, was used to prevent interaction between the Na⁺-pump and Src-kinase.

We have shown that a restricted K⁺, released via the intermediate-conductance Ca²⁺-activated K⁺-channel (IKCa) upon activation of endothelium, activates the α2 Na⁺-pump in VSMCs. This provides an endothelium-derived hyperpolarization (EDH). This signal is [Ca²⁺]o-dependent suggesting the involvement of Ca²⁺-sensing receptors. Elevation of [Ca²⁺]o, from 1 to 2.5 mM inhibits IKCa channels and hyperpolarization spreads mainly directly via myo-endothelium gap junctions. No role for Src-signaling was found in EDH.

Micromolar ouabain increased arterial sensitivity to noradrenaline and vasopressin. Ouabain had no effect on α2 Na⁺-pump downregulated arteries. Surprisingly, the arteries downregulated for the α2 Na⁺-pump had reduced sensitivity to agonists. Pharmacological inhibition of Src-kinase reduced also the arterial sensitivity and prevented potentiation action of ouabain.

VSMC synchronization was inhibited by ouabain via the α2 Na⁺-pump/NCX/Cx43 microdomain and a local raise of [Ca²⁺]o. Surprisingly, inhibition of Src-kinase had the effect opposite to ouabain and strongly improved intercellular coupling and VSMC synchronization.

Thus, we have shown that both Na⁺-pump/NCX microdomain and Src-kinase pathways modulate vascular contraction and VSMC synchronization, while the endothelium-dependent relaxation is independent from Src-kinase signaling.
Acidiﬁed vesicles of the endosomal-lysosomal system have regulatory functions in the kidney. The ClC-7 quadruple KO (Clcn7−/− or Clcn5−/−/−) mice show lysosomal storage disease like the KO, but their osteopetrosis is milder. Lysosomal pH is unchanged in either Clcn7−/− mice or Clcn5−/−/− mice because of a lysosomal cation conductance. Clcn7−/− and Clcn5−/−/− mice accumulate less Cl− than WT lysosomes, as also backed up by model calculations. Our studies show that proton-driven chloride accumulation is important all along the endosomal-lysosomal pathway and suggest an important, hitherto unknown role of luminal chloride concentration.

**SW03.S12-3**

Developing brain as an endocrine organ: secretion and endocrine action of dopamine

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It is generally considered that the development of the neuroendocrine system in ontogenesis begins with the ‘maturation’ of peripheral endocrine glands, which first are self-governed and then, after a full development of the brain, operate under the hypothalamic control. Nevertheless, the brain neurons begin to secrete neurohormones just after their origin and long before the establishment of the blood–brain barrier (BBB) and interneuronal synaptic relationships (synaptogenesis). Therefore, we hypothesized that the differentiating neurons function as secretory cells and the brain operates as an endocrine organ over the ontogenetic period from the neuron origin to the establishment of BBB and synaptic interneuronal relationships, in rats by the second postnatal week. The goal of this study was to test our hypothesis taking neuron-derived dopamine (DA) as a marker of the brain endocrine activity. First, it was shown that the DA concentration in plasma of rats from the 18th fetal day till the 3rd postnatal day, the whole studied period, was high enough for providing an endocrine action on the peripheral targets whereas DA was almost undetectable in plasma at the 30th postnatal day, after BBB closure. Then, it was proved that circulating DA was delivered from the brain by showing a drop of the DA concentration in plasma of rats at the 3rd postnatal day, after the BBB closure. From the above data, it follows that DA synthesized in the brain dopaminergic neurons is delivered to the general circulation providing an endocrine control of the pituitary prolactin secretion. After Rhodopsin-type GPCRs Adhesion class G protein-coupled receptors – a physiological riddle wrapped up in a signaling enigma

**SW03.S12-4**

The neuronal functions of Adhesion-class G protein-coupled receptors - a physiological riddle wrapped up in a signaling enigma

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After Rhodopsin-type GPCRs Adhesion class G protein-coupled receptors constitute the second largest group within the GPCR superfamily with over 30 receptor designs in humans.
The puzzling diversity of Adhesion-GPCR functions in immunity, development, neurobiology and tumor progression and their immense variability in N-terminal protein domains has been an obstacle to investigate the mechanism of Adhesion-GPCR activation on a molecular scale. The absence of known ligands and intracellular interactors in the past additionally complicated the development of informative assays probing Adhesion-GPCR function. We have devised a complementation assay using the nematode *C. elegans* as an *in vivo* test tube to determine the requirement of different receptor domains of the prototype Adhesion-GPCR Latrophilin/LAT-1 for phenotypic rescue of a latrophilin null allele. By virtue of assessing a large panel of receptor variants we find that the GPS (GPCR proteolysis site) motif functions as a tethered endogenous ligand for the 7TM-domain of Latrophilin/LAT-1. The GPS motif is part of the GAIN (GPCR autoproteolysis inducing) domain that constitutes the molecular hallmark feature present in all Adhesion-GPCRs. This indicates that the GPS/GAIN-7TM domain interaction is a pivotal step in the activation of individual if not all Adhesion-GPCRs.

**SW03.S12-5**

Propranolol restricts the mobility of single quantum-dot labelled EGF-receptors on the cell surface

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The epidermal growth factor receptor is a tyrosine receptor involved in morphogenesis, proliferation and cell migration. Its up-regulation during tumorigenesis makes this receptor an interesting therapeutic target. Here, we describe the effects of propranolol on the mobility of single receptors along the surface of the cell. To observe single receptors, they were labelled with quantum dots and visualized in a TIRF microscope. The single receptors showed a clear stop-and-go motion; their diffusive tracks were continuously interrupted by sub-second stalling events. Because the ratio between these stop and go events is approximately one, this leads to a significant underestimation of the actual diffusion coefficient of the receptor. When propranolol was added we found that: (i) the diffusion rate reduces by 20%, and (ii) the receptor gets frequently stalled for longer periods of multiple seconds. This reduced mobility of the receptor upon drug treatment may signal the first steps of the internalization of the receptor that will lead to its down-regulation.

**SW03.S12-6**

Effect of Lys-plasminogen on platelet functions

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Platelet surface serves as a site for assembly of proteins of the plasminogen activator system. It is thought that Glu-plasminogen circulating in plasma binds to platelet membrane and is converted firstly into Lys-plasminogen and then into plasmin. Plasminogen/plasmin sorption on the cell surface can make influence on the platelet properties. In the present work we have studied the influence of Glu-and Lys-form of plasminogen on the aggregation washed human platelets and their secretion of plasminogen activator inhibitor PAI-1.

Exogenous Lys-plasminogen inhibits human platelet aggregation in preparations of platelet rich plasma. We have shown that Lys-plasminogen but not Glu-plasminogen inhibits thrombin-and collagen-induced aggregation in preparations of washed human platelets. The level of aggregation in both cases decreases at least in two times. Inhibitory effect of Lys-plasminogen is observed during the second wave of aggregation, while platelet α-granules secrete their proteins. It has been found that l-α-aminoacaproyc acid (1 mM) abolished inhibitory effect of Lys-plasminogen that is compatible with the participation of 1–5 kringles of the proenzyme. Aprotinin, which is able to bind with the catalytic domain of plasminogen molecule, makes no influence on the inhibitory effect. Therefore, inhibitory action of Lys-plasminogen is not induced by the impurity of the proenzyme preparation and can’t be related with the effect of plasmin forming on the platelet surface. We have established that inhibitory effect of Lys-plasminogen on platelet aggregation doesn’t lead to change of the level of PAI-1 secreted by platelets during this process. It has been suggested that observed Lys-plasminogen effect is related with platelet-platelet interaction. Quite possible, that Lys-plasminogen interacts with certain proteins which are exposed on the platelet surface during aggregation (integrin IbIIIa, fibrinogen, thrombospondin or vitronectin) and make contribution to this process.

**SW03.S12-7**

The alteration of subplasmalemmal structure of hepatocyte during cytotoxicity and its prevention by sodium thiosulfate

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*Introduction:* Cytoskeleton is crucially involved in virtually all aspects of a cell’s life, including cell shape changes, cell division, cell movement contacts and signaling between cells, and dynamic transport events [1]. On the main causes leading to injury of cells can be structural changes occurring in the cytoskeleton of PM [2]. Defects in components of the cytoskeleton affect the ability of the cell to compensate at both functional and structural levels in the long term [3].

*Aims:* Understanding of the mechanism lying in the changes of subplasmalemmas will give opportunities for searching the medicaments assuring maximal protection of cells.

*Methods and Results:* The hepatocytes of Wistar rat liver at hepatotoxicity as well as at experimental crush syndrome were studied by the method of TEM under the electron microscope Philips CM 10. The structures which had ability to change their size in vertical and horizontal ways, as a response on the pathological influence were observed, as well as it was shown the heterogeneity of subplasmalemmal layer, which was presented by different structures. From the safeness of these structures as well as from the energy producer apparatus of cells (mitochondria) depends the response of hepatocytes on the investigated stress influence.

*Conclusion:* So, the alterations in the structure of observed subplasmalemmal structures as well as in energy producing apparatus of cells could be one of the main reasons leading to post stress influence damages of hepatocytes.

*References*


Abstracts

SW03.S12-8
Mammals aquaporin modulators: screening by heterologous expression in Saccharomyces cerevisiae
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Aquaporins (AQPs) are a family of transmembrane proteins ubiquitous in nature. These proteins form highly selective channels for water and, in the case of aquaglyceroporins, other small solutes such as glycerol and urea.

Thirteen different mammalian AQPs have been identified so far, present in almost all organs and tissues. It is clear that they play fundamental roles in human physiology and pathophysiology correlating with several diseases such as cancer. Therefore, AQP modulator drugs are predicted to be of broad medicinal utility. Although some compounds have been described as AQP inhibitors, few are considered to be suitable candidates for clinical development and none of them showed specificity for aquaglyceroporins.

Using a screening system based on permeability analysis of human red blood cells, we found a potent inhibition of the human AQP3 aquaglyceroporin by gold(III) complexes [1] that interestingly also possess anticancer properties in vitro. These compounds showed to be specific for AQP3 not affecting AQP1 also present in red blood cells. The high selectivity and nanomolar range efficacy of these compounds, together with their high water solubility, renders them promising lead drugs for future in vivo studies.

Nevertheless, there are numerous critical issues to be addressed before engaging in clinical studies such as the effect of these compounds on other mammalian AQPs as well as their modulation mechanism. To assist these questions, we are constructing a Saccharomyces cerevisiae deletion strain deficient in endogenous aquaporins and glycerol transporters to be used as heterologous expression system. This tool will enable to access AQP function individually, thus elucidating these questions, as well as to easily screen the effect of several other compounds as AQP modulators drugs.

Reference

SW03.S12-9
Calcium signaling recruits substrate transporters GLUT4 and CD36 to the sarcolemma without increasing substrate uptake
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Objective: Activation of AMP-activated protein kinase (AMPK) induces translocation of the glucose transporter GLUT4 and the long-chain fatty acid (LCFA) transporter CD36 from endosomal stores to the sarcolemma to enhance glucose and LCFA uptake into cardiomyocytes, respectively. However, it is unknown whether acute increases in [Ca2+]i stimulate translocation of GLUT4 and CD36 and uptake of glucose and LCFA as well, and whether Ca2+ signaling converges with AMPK signaling to exert these actions. This is relevant because Ca2+/calmodulin-activated kinase kinase-β (CaMKKβ) is positioned directly upstream of AMPK. Therefore, we studied the interplay between Ca2+ and AMPK signaling in the regulation of cardiomyocyte substrate uptake.

Methods: Primary cardiomyocytes were incubated with inhibitors or activators of Ca2+ signaling in the absence or presence of inhibitors or activators of AMPK signaling for assessing AMPK activation status, GLUT4/CD36 translocation, and glucose/LCFA uptake rates.

Results: Treatment of cardiomyocytes with Ca2+ signaling inhibitors did not affect AMPK-Thr172 phosphorylation and AMPK-mediated glucose/LCFA uptake, but induced GLUT4/CD36 translocation which was sensitive to Ca2+ signaling-inhibitors. Remarkably, Ca2+ induced nutrient transporter translocation was not accompanied by increased substrate uptake, unless cardiomyocytes were co-treated with AMPK activators.

Conclusion: Ca2+ signaling shows no involvement in AMPK dependent GLUT4/CD36 translocation and substrate uptake, but elicits transporter translocation via a separate pathway requiring CaMKKβ/CaMKs. However, Ca2+ induced transporter translocation by itself appears to be ineffective to increase substrate uptake, but requires additional AMPK activation to effectuate transporter translocation into increased substrate uptake. Ca2+-induced transporter translocation could play a role under conditions of supraphysiological energy demands.

SW03.S12-10
Effect of exogenous annexin A2 on proliferation and mineralization of human osteosarcoma cells
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Osteosarcoma (OS) is an aggressive, primary bone cancer affecting children and young adults. Unsatisfactory therapy for metastatic...
osteoosarcoma and conflicting results on the role of annexin A2 (AnxA2) in osteosarcoma progression justify the need for further studies of AnxA2 as a modulator of the aggressive phenotype of OS. In this report, we show that AnxA2 and its binding partner (S100A10) are selectively translocated into matrix vesicles as well as secreted into conditioned media by osteosarcoma cell lines undergoing mineralization. To elucidate the role of secreted AnxA2 in development of OS cell phenotype in vitro, we analyzed the expansion and the osteogenic potential of human OS cells (osteoblast-like Saos-2 cells and osteolytic, highly metastatic 143B cells) grown in the presence of human recombinant AnxA2 in the culture media at a final concentrations varying from 0.05 to 5 μg/ml. As a vehicle, OS cells were treated either with human recombinant AnxA6 or bovine serum albumin at the same concentration range as AnxA2. After 7 days of incubation, the cells were analyzed for their viability (MTT assay), proliferation rate and cell cycle (flow cytometry) and migration (confocal microscopy). The effect of exogenous AnxA2 on the mineralization potency of OS cells was determined by measuring alkaline phosphatase activity. Furthermore, AnxA2 distribution on the cell surface and its uptake by OS cells was observed using FITC-stained recombinant AnxA2. The results presented in this work suggest that secretion of AnxA2 to the extracellular matrix may have prognostic value for the monitoring of osteosarcoma progression. In addition, they reveal a contribution of exogenous AnxA2 in balance between mineralization and invasiveness of osteosarcoma cells that might be dependent on its Sl100 binding partners.

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**Sw03.S12-11**

**Membrane transporter bilitranslocase – structural model for transmembrane domains**

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We have predicted the trans-membrane domains of bilitranslocase [1], a transporter of bilirubin from blood to liver cells, by chemometrics methods [2] based on structural data of trans-membrane proteins available in PDBTM (http://pdbtm.enzim.hu/). NMR data were acquired to confirm helical structure of transmembrane segments [3]. The information about the 3D structure of membrane proteins is of great importance in the study of the protein transport mechanism; however, only the primary structure of bilitranslocase is available due to extreme experimental difficulties regarding its isolation or synthesis. Consequently, we have performed experimental studies on isolated 18-27 amino acid residues long segments of synthetic polypeptides [3] corresponding to trans-membrane domains predicted chemometrically [2]. A speculative transport mechanism including conformational changes of two trans-membrane segments at a proline-induced kink of the alpha helix is anticipated. Further studies will involve the analysis of interactions between the alpha helices of bilitranslocase that are spanning the membrane. Two transmembrane regions that contain proline kinks at equivalent position in the membrane hint towards allosteric nature of the protein.

**References**


**Sw03.S12-12**

**Mechanisms of the protein coat formation in the activated platelet subpopulations**

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**Introduction:** Physiological platelet activation leads to the formation of a procogulant subpopulation that is characterized by high level of phosphatidylserine, retention of α-granule proteins on the outer membrane surface and support prothrombinase activity. In spite of research of phosphatidyserine-expressing platelets at last decade the question about the mechanisms of formation and or retention on their surface of α-granule proteins remains open. The aim of this study was to investigate the mechanisms of protein coating on phosphatidyserine-expressing platelets.

**Methods:** Gel-filtered platelets activated by either thrombin with collagen-related peptide (CRP) or CRP under varying conditions, labeled with annexin-V, anti-human fibrinogen antibody and anti-human trombospodin antibody, were analyzed by flow cytometry Accuri C6.

**Results:** We found that fibrin(ogen) and trombospodin were localized on one and the same PS-expressing platelets. Besides, a fibrinogen-cleaning protease ancierson increased the amount of fibrin(ogen) and thrombospodin on the surface of the PS-positive platelets stimulated with CRP. Still, inhibition of fibrin polymerization did not suppress the trombospodin ‘coat’. Addition of tissue transglutaminas (tTG) led to increased levels of fibrinogen on the PS-positive platelets stimulated with CRP. Addition of a pan-transglutaminase inhibitor T101 or fibrin polymerization inhibitor GPRP decreased the tTG effect.

**Conclusions:** Thus, we can say that fibrin(ogen) authentically participates in the protein coat formation on the surface of phosphatidyserine-expressing platelets, while tTG is able, in principle, be participant in protein coat formation on the surface of these cells.

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**Sw03.S12-13**

**The use of fluorescent indicators to study the water and ion transport across plasma membrane of renal collecting duct principal cells**

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Kidney collecting duct principal cells play the key role in regulated tubular water and sodium reabsorption and potassium secretion. The results of investigation of transport processes through these cells are distorted due to the invasive nature of the
A PrPC-caveolin-Lyn complex inhibits GSK3βeta activity and potentiates serotonin release in serotonegenic neurons


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The prion protein was first identified under its scrapie isoform as the pathogenic agent responsible for Transmissible Spongiform Encephalopathies. This protein results from the pathogenic conversion of the host-encoded cellular prion protein (PrP\(^{\text{C}}\)), which is ubiquitous, GPI-anchored to the membrane, and particularly abundant in neurons. Despite much recent progress, our knowledge on the physiological role of PrP\(^{\text{C}}\) is still far from complete. We used appropriate fluorescent indicators to measure the dynamics of cell volume in hypotonic or Ca\(^{2+}\)-free medium and dynamics of intracellular concentration of sodium ions in low-sodium medium with sub-second time resolution. Experimental data were analyzed by the biophysical model of membrane transport. The combination of experimental approaches and modeling allowed to obtain quantitative estimates of plasma membrane permeabilities for water, sodium, potassium and chloride ions.

Also fluorescent dyes were applied for identification of membrane permeabilities of the same cell type isolated from rats subjected to the chronic salt loading. It was shown that increased NaCl intake caused more than threefold decrease of both potassium and sodium permeabilities.

To conclude, the current study reveals that the fluorescent indicators could be used to study transmembrane water and ion transport trough intact epithelial cells in dynamically changing extracellular environment.

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SW03.S12-15
Infertile HSL-knockout mouse testis shows class B scavenger receptor up-regulation, disrupted lipid raft microdomains, and activated p-ERK, p-AKT, and p-SRC

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There is a tight relationship between fertility and changes in cholesterol metabolism during spermatogenesis. In the testis, class B scavenger receptors (SR-B) (SR-BI, SR-BII, and LIMP II) mediate the selective uptake of cholesterol esters from HDL, which are hydrolyzed to unesterified cholesterol by hormone sensitive lipase (HSL). HSL is critical because HSL knockout (KO) male mice are sterile.

Objective: To determine the effects of the lack of HSL in testis on the expression of SR-Bs, lipid raft composition, and related cell signalling pathways.

Methods: WT and HSL-KO mice testis were frozen or embedded in paraffin to study protein expression and immunolocalization. Seminal fluid was obtained from epididymidis for spermatozoa counts and motility. Mice testis lipid rafts were isolated by sodium carbonate extraction and sucrose gradient fractionation.

Results: HSL-KO mouse testis presented altered spermatogenesis associated with decreased sperm counts, sperm motility and infertility. In WT testis, HSL is expressed in elongated spermatids, SR-BI in Leydig cells and spermatids, SR-BII in spermatocytes and spermatids but not in Leydig cells, and LIMP II is present in Sertoli and Leydig cells. The lack of HSL in mouse testis induces augmented expression of SR-BI, SR-BII, and LIMP II, alters caveolin-1 localization in lipid raft plasma membrane, and induces activation of key proteins (p-ERK, p-AKT, and p-SRC) in cell signaling pathways involved in differentiation, proliferation, and other cellular processes during spermatogenesis.

Conclusions: The lack of HSL dramatically disrupts cholesterol homeostasis in the testis, with augmented expression of SR-Bs (SR-BI, SR-BII, and LIMP II), altered lipid raft microdomains, and activation of different signaling pathways with relevance in spermatogenesis.

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Membrane cholesterol oxidation and depletion effects on synaptic vesicle cycle in frog motor nerve terminals
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In experiments on frog (Rana ridibunda) neuromuscular junction the influence of cholesterol oxidation and depletion by methyl-β-cyclodextrin (MCD) on the presynaptic vesicular cycle was investigated. Application of cholesterol oxidase (1 u. a.) during 1/2 h led to the oxidation of ~0.007 mg cholesterol per g tissue and reduced stability of lipid rafts in the nerve terminals. It was shown that 1 mM methyl-β-cyclodextrin (MCD) which reduced membrane content of cholesterol on ~15% also decrease raft stability at presynaptic membrane. Using electrophysiological techniques it was shown that the cholesterol oxidation and depletion decreases the evoked neurotransmitter release. In experiments with fluorescent FM-dyes the depression of the synaptic vesicles exo-endocytosis were revealed. In case of cholesterol oxidation comparative analysis of electrophysiological and optical data, as well as experiments with water soluble quencher of FM-dye indicated the possibility of some neurotransmitter release by «kiss-and-run» pathway, when short-lived fusion pore is formed. The cholesterol depletion from external membranes and membranes of recycling synaptic vesicles in addition to above described effects blocks processes of endocytosis. It was concluded that cholesterol oxidation and depletion inhibit evoked exocytosis, and also synaptic vesicle delivery from reserve pool to sites of exocytosis. Perhaps the synaptic vesicles of recycling pool release the neurotransmitter using the kiss-and-run mechanism in condition of cholesterol oxidation.

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Effects of EPA and DHA on interleukin 6 and adiponectin secretion by 3T3-L1 cells
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Obesity is associated with increased immune activation and chronic state of low-grade inflammation in adipose tissue. Numerous recent studies have revealed that in obesity secretion of proinflammatory cytokines (interleukin 6, TNF Alpha, MCP – 1, leptin) is increased, and anti-inflammatory cytokines (adiponectin, interleukin 10) secretion is decreased. These dysregulations are strongly connected with development of insulin resistance. The n – 3 PUFA, especially EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) have been reported to have protective effects in many types of chronic inflammatory conditions, such as: rheumatoid arthritis, asthma, Crohn’s disease and psoriasis. They also improve lipid metabolism, prevent obesity and diabetes.

The aim of the present study was to evaluate the effects of EPA and DHA on interleukin 6 and adiponectin secretion by 3T3-L1 cells. Differentiated 3T3-L1 adipocytes were cultured for 48 h in the presence of 100 μM EPA, or 50 μM DHA complexed to albumin, whereas in control conditions only albumin was added to the medium. The concentration of adipokines (interleukin 6 and adiponectin) in conditioned media was measured using mouse-specific ELISA kits.

ELISA tests revealed that both polyunsaturated fatty acids increased the concentration of secreted adiponectin compared with the control (58% and 35%, respectively). Moreover, EPA supplementation increased interleukin 6 concentration in conditioned medium, while DHA exerted an opposite effect (45% and 28%, respectively).

The statistical significance of differences between groups was determined by the Student's t-test (two-tailed). The results were considered significant when the p-value was <0.05.

In summary, the investigated polyunsaturated fatty acids: EPA and DHA affected the secretion of pro- and anti-inflammatory cytokines by adipocytes. Action of docosahexaenoic acid seems to be anti-inflammatory, however, on the basis of the obtained data it was not possible to determine whether eicosapentaenoic acid exerts anti- or proinflammatory properties.

Senescent cells impact their microenvironment by direct protein transfer
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Cellular senescence, a permanent cell-cycle arrest, is an intrinsic barrier against tumorigenesis. It also limits tissue damage response. However, senescent cells can promote tumorigenesis, inflammation and tissue damage in their microenvironment. Senescent cells accumulate in tissues with age and can promote tissue ageing. The soluble factors and extracellular matrix secreted from the senescent cells were reported to execute these cell non-autonomous phenomena. Here we show a novel mechanism that senescent cells use to affect the neighboring cells – the direct protein transfer. Our results demonstrate that proteins are preferentially transferred from senescent cells to natural killer cells and to cancer cells. The transfer is strictly depended on cell contact and actin polymerization. Moreover, the transferred proteins lead to functional changes in the recipient cells. We propose that Tunneling Nanotubes (TNTs) is a mechanism that accounts for direct protein transfer from senescent cells. Indeed, we detected TNTs structures between senescent cells and the neighboring cells. Thus, our results reveal a novel mode of communication between senescent cells and their microenvironment. Unraveling of the mechanism of the protein transfer from senescent cells might lead to better understanding of tumorigenesis and tissue ageing.

Role of transmembrane ion and water transport in outer medullary collecting duct principal cells volume regulation in the diuretic state
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The problem of maintaining a constant volume under conditions of extracellular osmotic pressure fluctuations is particularly relevant for transport epithelium cells, such as renal collecting duct cells. The aim of the present work was to investigate the transmembrane ion and water transport in rat outer medullary collecting duct (OMCD) principal cell in hypotonic medium. Hypotonic shock was created by PBS diluted twofold with distilled water. The changes in the cell volume in the course of response to osmotic shock were studied by the method based on the effect of Calcein fluorescence quenching by cytoplasmic proteins. Intracellular sodium concentration was measured using the fluorescent dye Sodium Green. The influence of diet with high content of NaCl on Na transmembrane transport was studied. Diet courses
3.3 fold decrease of plasma membrane sodium permeability. To clarify the role of cotransporters in OMCD principal cells, we studied effects of KCC1 and NKCC1 inhibitors. In isotonic medium the presence of inhibitors did not considerably affect the transmembrane transport in OMCD cells. The inhibitor of KCC1 DIOA decreased the rate of regulatory volume decrease (RVD) in hypoosmotic conditions. The OMCD cells after hypoosmotic shock demonstrate dramatic decrease of water permeability. It was found, that hypotonicity significantly decreased the content of AQP2, but not AQP3, in membrane fraction of rat renal outer medulla slices. This result indicates that internalization of AQP2 is one of the important intercellular processes involved in RVD. It was observed that the OMCD cells of heterozygous vasopressin-deficient Brattleboro rats are less adaptive to hypoosmotic challenge compared with water loaded Wistar rats. It was suggested that lack of endogenous vasopressin in Brattleboro rats courses the permanent changes in the expression or function of transporters that are important for RVD in OMCD principal cells.

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**SW03.S12-20**

**Role of PKC in antidiuretic action of vasopressin**

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The PKC activity and calcium-dependent second messengers seem to be important for regulation of water permeability of kidney collecting duct epithelia by vasopressin. Inhibition of PKC impaired vasopressin dependent increase of plasma membrane water permeability of principal cells. Microdissected fragments of rat kidney outer medulla collecting duct (OMCD) were used in the study. In experiments PKC was inhibited by 100 nM of Ro-31-8220. Coefficient of osmotic water permeability of total cell surface (Pf) was calculated from the initial rate of cell swelling following the osmotic shock caused by changing the medium osmolality from isotonic to hypotonic (300–150 mOsm/kg). In our experiments agonist of vasopressin receptor V2 desmopressin (dDAVP 1 nM) increased the Pf in from 124 ± 9.5 to 192 ± 15.8 mm/s. Incubation of cells with dbcAMP (N(6),2′-O-dibutyryladenosine 3′5′ cyclic monophosphate 10 mM) also led to increasing of water permeability from 129 ± 10.1 to 197 ± 16.2 mm/s. Chelating of Ca2+ by intracellular chelator BAPTA abolished the increase of water permeability as response of dDAVP and dbcAMP action. Inhibition of PKC suppressed the desmopressin-stimulated increase of plasma membrane water permeability. Hydrophobic derivative of cyclic AMP, db-cAMP increased water permeability of plasma membrane even in the presence of PKC inhibitor.

We suppose that PKC play important role in the mechanism of vasopressin signal transduction on the step from V2 receptor to Adenylate Cyclase. Vasopressin induced increasing of intracellular calcium concentration has critical importance for regulation of water permeability by vasopressin.

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**SW03.S12-21**

**Intracellular trafficking of EGFR in response to binding with soluble heparin-binding EGF-like growth factor**

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Activation of EGFR family members: EGFR and ErbB4 promotes survival, proliferation and migration in different cell types. Ligand binding to EGFR results in EGFR activation and further recycling or lysosomal degradation of ligand-receptor complex. In addition, EGFR and its ligands: EGF, TGF-α and amphiregulin has been detected in nucleus of cells with highly proliferating activities. Another EGFR ligand – sHB-EGF acts as a cell proliferation and cell survival factor in cancer cells. Therefore, sHB-EGF may play an important role in tumor progression. Nevertheless, there is no evidence on the nuclear localization of sHB-EGF and its ability to induce EGFR nuclear importation.

In this study, we demonstrated nuclear importation of EGFR/sHB-EGF ligand-receptor complex via retrograde transport and its followed association with promoter region of cyclin D1 in A431 cells. It was also shown that EGFR kinase activity is strongly required for induction of internalization and further nuclear translocation of EGFR/shHB-EGF ligand-receptor complex. Therefore sHB-EGF might promote nuclear importation of EGFR to activate genes required for highly proliferating activities.

**SW03.S12-22**

**Detrimental effect of bacterial lipopolysaccharide on vasotonin-induced osmotic water permeability; possible role of iNOS expression and changes in triacylglycerol metabolism and oxygen consumption**

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1, E. Lavrova1, S. Nikolaeva1, E. Fock1, E. Fedorova1, V. Bachtueva1, S. Herterich2, S. Gambaryan1 and I. Braitlovskaya1

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induced OWP in a cGMP-dependent manner suggesting the involvement of LPS-triggered endogenous NO to inhibition of OWP. Also, LPS caused a reduction of oxygen consumption by FUBEC and decreased metabolic activity determined by MTT test. FACS analysis of cells stained by Nile Red fluorescent lipid dye revealed that LPS treatment increased amount of neutral lipids in FUBEC lipid droplets. Using FUBEC loaded with [3H]-oleic acid we showed that this effect of LPS is related to time-dependent decreased lipolysis of lipid droplets-associated triacylglycerol and oxidation of free fatty acids suggesting the involvement of LPS-induced changes in the expression/activity of TAG-lipases or mitochondrial β-oxidation enzymes. Taken together, these data demonstrate that both increased iNOS-dependent NO production and decreased availability of free fatty acids for energetic metabolism might contribute to the detrimental effect of LPS on water transport function of FUBEC.

### SW03.S12-23

**Adenosine induces markers of epithelial to mesenchymal transition in renal proximal epithelial tubule cells**

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**Introduction:** The transition from epithelial to mesenchymal phenotype (TEM) was evidenced in HK2 renal tubular cells knocking down for the equilibrative nucleoside transporter 1 (ENT1). Further, kidneys isolated from ENT1 knockout mice showed higher levels of interstitial collagen and α-SMA-positive cells. These observations link altered adenosine handling with pro-fibrotic TEM. Interestingly, increasing levels of adenosine have been quantified in plasma of patients affected by diabetic nephropathy (DN) and in urine from experimental models of diabetes. Our aim was to study the effects of high glucose concentrations on extracellular adenosine availability and the role of adenosine receptor in the transdifferentiation of renal tubule epithelial cells.

**Methods:** HK2 cells were exposed to adenosine (10 μM), the general agonist of adenosine receptors NECA (5 μM), the α-agonist TGF-β (5 ng/ml) and glucose 5 or 25 mM for 24 h. The TEM was evaluated by detecting the expression of α-SMA and fibronectin. Similarly, the expression of the ecto enzyme CD73 and ENTs was evaluated by western blots. The activity of ENTs was quantified by measuring [3H]-adenosine uptake. The 5′-ectonucleotidase activity was evaluated quantifying AMP hydrolysis in exposed cells by HPLC.

**Results:** Total uptake activity mediated by ENTs was decreased in HK2 cells exposed to high glucose concentration. Particularly, the activity of ENT1 was reduced by this condition. The 5′-nucleotidase activity mediated by CD73 was not affected by the exposure of cells to 25 mM glucose. Adenosine, NECA and TGF-β increased the expression of phenotypic transition markers α-SMA and fibronectin in HK2 cells. The TEM induced by NECA can be blocked by using an antagonist of the adenosine A3 receptor subtype.

**Conclusions:** Adenosine is an inducer of TEM in HK2 cells which could be relevant in the progression of DN where the levels of the nucleoside are increased. The induction of TEM by adenosine was mediated by the low affinity A3 receptor subtype. The lower uptake activity of adenosine mediated by ENT1 seems to be the main contributor to raise the levels of the nucleoside under high glucose exposure, locally.

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### SW03.S12-24

**Transport of platinum-based anticancer drugs by recombinant human copper ATPases (ATP7A/B)**

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ATP7A and ATP7B are members of the P1P-ATPase subfamily of the P-type ATPases, and catalyze the ATP-dependent copper transport across cellular membranes via a process involving formation of a phosphoenzyme intermediate [1]. Numerous physiological processes depend on adequate and timely copper transport by these proteins. Inactivation of either ATP7A or ATP7B is associated with severe metabolic disorders, known as Menkes (ATP7A) and Wilson (ATP7B) diseases.

Platinum-based anticancer drugs, e.g. cisplatin, carboplatin and oxaliplatin, are used to treat various types of tumors. As with most anticancer drugs, the efficacy of treatment is limited by intrinsic and acquired resistance of tumor cells. Recently, experimental evidence indicated that Cu-ATPases mediate resistance to platinum anticancer drugs [2]. However, the mechanism by which Cu-ATPases mediate resistance to such drugs is not fully understood.

The present study aims at investigating the mechanism of interaction of platinum drugs with recombinant human ATP7A/B, obtained by heterologous expression in COS-1 cells [3]. We performed ATP concentration jumps on microsomes containing ATP7A or ATP7B adsorbed on a solid supported membrane [4,5]. The ATP jump induces a current transient in the presence of platinum-based drugs. We suggest that the observed current transient is due to movement of platinum through ATP7A/B upon ATP utilization (formation of the phosphoenzyme intermediate required). We also found that copper interferes with platinum transport and vice versa. A mechanism for the electronic platinum transport by ATP7A/B is proposed.

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**References**


### SW03.S12-25

**Study of the role of extracellular adenosine on chemoresistance in glioblastoma stem-like cells**

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**Background:** Glioblastoma multiforme (GBM) is a highly malignant primary brain tumour. Consistent studies have shown glioblastoma stem-like cells (GSCs) to be the only tumorigenic
population and responsible for the extreme chemoresistance in this tumour. This important finding supports a key role for GSCs in progression, recurrence and probably tumour genesis. An essential obstacle for chemotherapy of GBM is the presence of multiple drug resistance (MDR) transporters, essentially Mrp1. To date, there is a limited number of multiple drug resistance studies in GSCs. The activity of ecto-5'-nucleotidase (CD73) is essential for the production of extracellular adenosine and there is a correlation between the overexpression of CD73 and MDR transporters in some cells. Our main objective was to determine the role of CD73 and adenosine receptors on the regulation of Mrp1 in GSCs.

Observations: We have found that the intrinsically extreme chemoresistance mediated by Mrp1 in GSCs can be reversed by blocking the Akt/mTOR signaling pathway. Interestingly, we identify a high expression of CD73 and adenosine A3 receptors in GSCs compared to differentiated bulk GBM population cells. Concurrently, CD73 mediated AMPasa activity was found notably increased in GSCs. Moreover, using a specific inhibitor of CD73 activity (AOPCP) or an adenosine A3 receptor antagonist (MRS1220), we observed a significant decrease in the expression and activity of Mrp1. The viability of GSCs was decreased by 50% upon inhibition of the activity of CD73 together with the exposure to antitumor drugs substrates of Mrp1, such as vincristine, etoposide or taxol.

Conclusions: It is urgent today, to evaluate new therapeutic alternatives for recurrence of GBM. In this context, a feasible way of chemo-sensitizing GSCs could be the inhibition of CD73 activity or the use of adenosine A3 antagonists to reverse the extreme refractoriness to anticancer drugs mediated by Mrp1, in this tumor.

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SW03.S12-26

The technique of spectral precision distance microscopy (SPDM)

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Progress in optical microscopy opens up new possibilities to overcome Abbe's diffraction barrier in conventional far-field microscopy. Here, we present a unique method of super-resolution microscopy which allows to study tiny structures such as viral particles as well as intracellular distributions of proteins and nucleic acids at the level of single molecules. The technique of Spectral Precision Distance Microscopy (SPDM) in photo activation mode works with commercially available fluorophors (e.g. Alexa-, Cy-, Atto-) which offer a suitable time-lapse recording of distinct blinking events in fixed specimens.

Self-developed software based on Matlab was used to reconstruct the SPDM images from the time series and to analyze the distribution and clustering of molecules of interest. Our method does not depend on the use of UV irradiation to switch the spectral properties of fluorophors.

SPDM photo activation in wide-field mode simplifies sample preparation and allows not only the observation of structures in close proximity to cell membranes as well as general intracellular structures such as within the nuclei.

Due to the extremely stable construction of our microscope we could record images for extended time periods leading to a significant improvement in spatial resolution in comparison to previous SPDM set-ups.

In the current system, we established a novel beam shaping procedure and a high-precision optical alignment using a Shack Hartmann Sensor. We also improved the thermal and mechanical stability of the entire optical system.

We have applied SPDM for resolving various biological questions, e.g. to determine the exact localization of the GADD45a protein inside the nuclear envelope. Conventional confocal microscopy studies revealed the location of GADD45a in the nuclear envelope of about 10–20% of cells. However, they did not allow us to decide whether GADD45a is located on the inner or the outer nuclear membrane which are 40–80 nm distant from each other. With SPDM, we observed that Gadd45a is located in clusters on the same level or outside of the outer nuclear membrane. Our studies prove that SPDM can be efficiently used for resolving multiple complex subcellular structures on the molecular level.

Microtubules in culture mammalian cells often, but not always organize radial array. Microtubule radial array can converge to compact microtubule organizing centre (MTOC) represented by centrosome and/or Golgi apparatus (Golgi). We decided to compare vesicular transport in cells with radial and with chaotic microtubules. Vero green monkey kidney cells displayed radial microtubules, and the array was disturbed after LOSK/SLK kinase inhibition. We traced the movement of ERGIC (endoplasmic reticulum-Golgi intermediate compartment) vesicles marked with ERGIC53-GFP fused protein, in control Vero cells and in cells with inhibited LOSK/SLK kinase. It turned out that instant velocity of vesicle movements, estimated as their average displacement in one second, was unalterable. Disposition of vesicle movement distribution by their instant rates was also permanent. However, long-distance vesicular transport was affected after microtubule chaoticization: the rate of ERGIC assembly after its dispersion by Brefeldin A was significantly decreased in cells with inhibited LOSK/SLK. This effect could be explained apparently as a direct consequence of disturbed radial microtubule array.

Both Vero and BS-C-1 culture cells originated from green monkey kidney and both exhibit radial microtubule array. We found that Golgi had an enhanced capability to nucleate microtubules and to anchor them in BS-C-1 than in Vero cells. After total collapse of Golgi achieved either with treatment with Brefeldin A or by expression of dominant-negative Sar1A[T39N] Vero cells showed less relevant alteration in microtubule cytoskeleton than in the case of cells BS-C-1. On the other hand, LOSK/SLK inhibition which influences centrosome activity had more evident effect to microtubules in Vero than in BS-C-1 cells. We estimated that BS-C-1 cells had an increased expression of CLASP2 protein. However, CLASP2-GFP expression in Vero cells did not lead to increase of its Golgi microtubule-organizing activity. Probably, BS-C-1 cells had enhanced activity of GSK-3β protein kinase which participates in phosphorylation of CLASP2. We found also that inhibition of dynein activity (by expression of coiled-coil fragment of p150Glued dynactin subunit) did not lead to significant loss of microtubule nucleation events on the vesicles of dispersed Golgi. These data suggest that distribution of microtubule-nucleating factors to Golgi membranes may occur by dynein-independent manner and may depend on GSK-3β activity.

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SW03.12-28
Investigation of the cellular mechanisms underlying the carboxypeptidase E mutation
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Carboxypeptidase E (CPE) is an enzyme expressed in endocrine cells and peptidergic neurons functioning as both an exopeptidase and a sorting receptor. Recently, it has been reported that CPE plays a role in preventing neuronal cell death in the CA3 hippocampus so as to maintain normal cytoarchitecture for optimal electrophysiological and cognitive function in the adult brain. Studies on CPE-knockout mice showed total degeneration of neurons in the CA3 region of the hippocampus in adult mice 4 weeks of age and older. Additionally, increased CPE expression was involved in protecting hippocampal neurons from oxidative stress-induced apoptosis. The neuroprotective role of CPE prompted us to search for possible mutations in the human CPE gene that might be linked to human neurodegenerative diseases. A non-redundant nucleotide sequence database search with the human CPE nucleotide sequence as queries identified an EST that let us hypothesize that the anticonvulsant effect of ERK1/2 inhibition is associated with increased synthesis and exocytosis of GABA. Thus, obtained data demonstrated direct correlation of neurohormone/neurotransmitter exocytosis operated by SNARE complex with ERK1/2 activity levels.

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SW03.12-29
ERK1/2 kinase regulates exocytosis of neurohormones and neurotransmitters
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Synaptic exocytosis machinery is characterized by vesicles fusion at plasma membrane, which is mainly regulated by SNARE proteins, and it is the basis for neurotransmitter or neurohormones release. On the other hand it was shown that exocytosis can be regulated in a Ras/ERK dependent manner. We hypothesized that ERK1/2 can directly regulate SNARE proteins thus participating in neurotransmitter or neurohormones release. Firstly, we have analyzed vasopressin (VP) secretion machinery. Dehydration is well known factor, which activates VP system of hypothalamus. In this work we analyzed effects of dehydration induced by water deprivation on activities of ERK1/2 and SNARE in VP neurons. Our data demonstrated that the activation of VP transport and release was associated with the accumulation of the active ERK1/2 kinases and decreased SNAP25 levels in VP terminals of the posterior pituitary. Additionally, we have also analyzed a correlation of SNARE proteins with the development of epileptic seizures. It is known that epilepsy characterized with disruption of inhibitory and upregulation of excitatory neurotransmitter release. In our experiments audiogenic Krushinsky-Molodkina (KM) rats were recruited. Analysis of SNARE proteins demonstrated that in the temporal cortex and in the inferior colliculus the levels of SNAP25 and synaptobrevin2 were significantly lower in KM rats compared with Wistar rats. Moreover, KM rats were also characterized with increased content of phospho-ERK1/2 and diminished expression of GAD67 and NR2B. Also we observed colocalization SNAP25 and synaptobrevin2 with ERK1/2. Injections of MEK1/2 selective inhibitor SL327 led to abolition of seizure activity in 70% of KM rats, which were exposed to acoustic stimulation. Inactivation of ERK1/2 kinase activity was accompanied with increased GABA and NR2B expression in the neurons of temporal cortex and hippocampus that let us proposed that the anticonvulsant effect of ERK1/2 inhibition is associated with increased synthesis and exocytosis of GABA. Thus, obtained data demonstrated direct correlation of neurohormone/neurotransmitter exocytosis operated by SNARE complex with ERK1/2 activity levels.

In the mammalian brain MCH is expressed in neurons present in the hypothalamus, predominantly in the lateral hypothalamic area (LHA) and the zona incerta (ZI). Melanin-concentrating hormone (MCH) is a cyclic heptadecapeptide of multifarious biological functions. Its primary role in fish is the regulation of skin colour, whereas in mammals its orexigenic nature is of great importance. MCH expressing neurons regulate food intake and energy expenditure, and presumably influence other physiological functions, such as sleep and wakefulness as well.

In order to get further information on the intracellular traffic of MCH, immunohistochemical studies had been undertaken in the mouse brain.

Cell bodies and processes equally contain MCH (cf. Hazai et al. 2008, Brain Res.). We present here our recent observations on the colocalization of MCH with dendrite-specific and axon-specific markers, respectively, by using double-labeling immunofluorescence technique. The presumed diurnal variation of MCH has been investigated by comparing the immunoprecipitates densities in coronal brain sections of mice after staying 12 h in darkness with those after staying 12 h under luminous conditions. Immunoreactive cell bodies and processes had been both taken into account. MCH level in LH has been significantly higher (p < 0.005) at the end of
the light period, as compared to the end of the dark period, whereas no difference had been observed in the ZI. This experiment indicates that the equilibrium of the synthesis and transport of MCH in the course of the diurnal cycle is differently set in these two hypothalamic areas.

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**SW03.S12-31**

**Insulin receptor-related receptor is involved in renal bicarbonate secretion**

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Acid-base balance is critical for normal functioning of organisms. It is known that intercalated cells (ICs) are important for renal acid-base regulation. It was previously shown that insulin receptor-related receptor (IRR) is one of the molecular markers of β-intercalated cells (β-ICs). Recently it was found that IRR, an orphan receptor tyrosine kinase of the insulin receptor family, is an alkali sensor.

To analyze the possible role of IRR in the regulation of acid-base balance knock-out mice (KO) with targeted inactivation of the insrr gene encoding IRR were used. For the KO phenotype analysis blood and urine parameters were measured at normal conditions as well as at alkali load. It was found that knock-out mice have increased pH and bicarbonate levels in the blood at normal conditions. Mice with inactivated IRR developed metabolic alkalosis with impaired urinary bicarbonate secretion in response to alkali load. As contrast to wild type mice, IRR KO mice did not excrete the excess amount of bicarbonate to urine from the blood. Instead blood pH and bicarbonate level were increased. Dysfunction of IRR impairs the correct renal response reactions to metabolic alkalosis. Being a marker of β-ICs, IRR can have an impact on adaptation to alkalosis of intercalated cells. With IRR being disrupted, impaired β-ICs response to alkali load of the KO mice may be related with the alteration of number of the ICs. To test the hypothesis we made immunohistochemical staining of the cryo-sections of both KO and WT mice kidneys. Antibodies for Cl⁻/HCO₃⁻ anion exchanger (AE1) and pendrin as molecular markers of α-ICs and β-ICs respectively were used. No change in number of ICs between WT and KO mice at alkali load was found. It is correct to assume that regulation of the acid-base balance may be dependent not only of the total amount of the ICs but also on the secreting capabilities of each individual ICs. Alkali load of the IRR KO mice resulted in decreased both pendrin and proton pump expression of β-ICs. The observed decrease of pendrin expression as main bicarbonate transporter of β-ICs correlates with the aforementioned impaired renal adaptation to metabolic alkalosis.

**SW03.S12-32**

**Reduced surface expression and blunted pH-dependent gating of CIC-Kb chloride channel in mild Bartter syndrome caused by CLCNKB mutation within the selectivity filter**

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**Background:** CIC-Kb, encoded by CLCNKB gene, is a chloride channel belonging to the CIC gene family. It is expressed in the renal tubule, at the basolateral membrane, as well as in the inner ear (Ulchida S., J Clin Invest, 1995). Mutations occurring in the CLCNKB lead to the development of the Bartter syndrome type III (BS), a rare autosomal recessive disorder characterized by salt-wasting tubulopathy. Up to date, several mutations have been described as loss-of-function mutations and, in patients, may produce strong to mild clinical phenotype.

**Aim:** Functional consequences of a Valine to Methionine substitution at position 170 detected in eight patients displaying a mild Bartter syndrome phenotype.

**Methods:** Electrophysiological analysis using Two-Electrode Voltage-Clamp, surface expression with luminescence assay and Western-blot assay were used to characterize CIC-Kb mutant expressed in X. laevis oocytes.

**Results:** Conductance and surface expression are reduced by ~40% (13 and 60 oocytes respectively) for the CIC-Kb V170M mutant as compared to CIC-Kb WT. Western-blots also show that V170M total protein content is less abundant than WT. To functionally characterize the V170M mutant we investigated the sensitivities to external H⁺ and Ca²⁺, two biophysical properties of CIC-Kb. We observed a dramatic alteration in half-maximal pH inhibition (pKa), from pH 7.60 ± 0.05 (31) for the WT to pH 6.00 ± 0.11 (13) for V170M. The stimulating effect of external Ca²⁺ ([Ca²⁺]ext) was abolished for V170M mutant at pH 7.4 but could be restored at low pH (pH 6.0). Relative permeabilities experiments show that V170M mutation did not change the anion selectivity sequence when compared to the CIC-Kb WT. CIC-Kb bears another Valine in position 166, where other ClC proteins harbor a glutamate crucial for channel gating. Artificial mutations in this position had only moderate functional consequences, emphasizing the specific importance of Valine in position 170.

**Conclusion:** CIC-Kb V170M mutant showed an altered pHₐₜ and [Ca²⁺]ₜₐₜ sensitivity at physiological pH. This feature can partially explain the mild phenotype of BS. For this mutant, the partial impairment of plasma membrane targeting is compensated by a higher activity of the mutated channel under physiological conditions.

**SW03.S12-33**

**Differential regulation of ABCA1 in K562 cells by various differentiating stimuli**

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The K562 cell line is one of the best-studied model examples of human myeloid differentiation in vitro. These cells, derived from
a female erythroleukemia patient nearly 40 years ago, carry the Philadelphia chromosome with the transforming fusion protein BCR-ABL. They resemble common progenitors of the erythroid and myeloblastic lineage and can be experimentally differentiated into more phenotypically advanced cells by application of specific stimuli. These stimuli direct these cells into an erythroblast-like phenotype, with several studies showing the separate involvement of distinct pathways and transcription factors which seem to act in concert in physiological erythroid differentiation in vivo, such as GATA-1, KLF1 or NFE2.

ABCA1 is a large transmembrane protein from the ABC superfamily of ATP-dependent transporters. It is a key regulator of cholesterol trafficking from cells to lipoprotein complexes, especially from macrophages to apolipoprotein A1. Mutations in the ABCA1 gene cause Tangier disease, a familial hypoalphalipoproteinemia with an accumulation of cholesterol-loaded foam cells. Many studies have been performed on the mechanisms of transcriptional regulation of the ABCA1 gene – however, the ABCA1 protein is most probably absent from mature human erythrocytes and no involvement of the gene in erythroid differentiation has ever been reported.

We report here for the first time the significant level of expression of ABCA1 in the K562 cell line and induction of its expression at the transcriptional level by treatment of cells with some standard erythroid differentiation-inducing compounds, such as hemin or BCR-ABL inhibitors. In contrast, some other differentiating chemicals (most notably the anti-cancer drug cytarabine), while displaying many phenotypical and transcriptional hallmarks common with the previously mentioned group, were unable to induce the expression of ABCA1, pointing to a divergence in mechanism of this induction with regard to the core differentiation machinery. Moreover, there were significant temporal and mechanistic differences between the effect of hemin and BCR-ABL inhibitors on ABCA1 expression, which we used to dissect the presence of two separate induction mechanisms.

**SW03.S12-34**

**Transcription factor Cp2L1 controls cell fate decisions in the collecting duct**

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Kidney epithelia derive from metanephric mesenchyme by a process that includes epithelial conversion followed by specification of different epithelial cell types in different segments of the nephron. Little is known about transcriptional mechanisms that regulate cell identity in the developing nephron.

To identify genes that regulate specific cell types, we created an in vitro model of nephrogenesis. We incubated rat metanephric mesenchyme with growth factors (LIF and FGF) that we had purified from embryonic epithelia, and observed the time course of epithelial induction using microscopy and gene expression profiling. We identified 70 transcription factors that were regulated during epithelial conversion. We next over-expressed each of these factors in the metanephric mesenchyme using adeno-viral mediated gene transfer and found that only one transcription factor induced conversion, the Grainyhead family member, Cp2 L1. Grainyheads had been found to regulate the epithelial phenotype supporting the identification of Cp2 L1.

We confirmed that Cp2 L1 was expressed by developing epithelia of the nephron. Cp2 L1 was expressed by ‘distal nephron’ including the distal part of the S-shaped body, and in the adult, the Loop of Henle, the Distal Convoluted Tubule and cortical collecting ducts. To investigate its activity, we compared ChIP-seq from wild type kidneys with gene expression data from global Cp2L1 knockouts. The comparison yielded 82 genes that were significantly downregulated in the knockout, and contained Cp2 L1 binding sites within 10KB of the transcriptional start-site. Most striking, genes that are classically associated with kidney intercalated cells were bound and regulated by Cp2 L1. In the knockouts, we observed downregulation of major markers of alpha- and beta-intercalated cells including V-ATPase, Pendrin and AC 1 and other intercalated cell regulators, and instead found a monotonous pattern of principal cell-like epithelia. In fact, the pattern of Notch signaling was disrupted in the knockout.

**SW03.S12-35**

**The influence of the plant flavonoids on the domain shape in unilamellar vesicles**

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Flavonoids are a class of bioactive plant polyphenols. Some of them, in particular phloretin, reduce temperature of a melting of an individual lipid in unilamellar vesicles (UVs) [Cseth et al., Eur. Biophys. J., 2000]. The present study is an attempt to visualize the likely effect of the flavonoids on the laterally phase separation in a binary lipid mixture by the confocal microscopy. UVs were formed by the electroformation method at 25°C in 0.5 M sorbitol aqueous solution from dioleoylphosphatidylcholine: brain sphingomyelin in a molar ratio 80:20. Resulting UVs suspension contains 0.8 mM lipid. The lipid composition produces the lateral heterogeneity detectable by the lissamine rhodamine dipalmitoylphosphoethanolamine, fluorescence dye which prefers liquid disordered phase. So other phases remain colored. Under confocal microscopy at 25°C UVs have strictly irregular uncolored domains. The biochanin A or phloretin addition to the suspension up to 400 μM leads to appearance in UVs right circular domains. And at the same time 400 μM myricetin remains all domains with irregular shape. The mechanism of the influence of biochanin A and phloretin on the shape of domains is discussed.

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**SW03.S12-36**

**The electroneutral NaCl transport in the intercalated cells of the collecting duct is upregulated in PHAII-mutant WNK4 mice**

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**Objectives:** Gordon’s syndrome [also known as Familial Hyperkalemic Hypertension (FHH), or Pseudohypondostension type II (PHAII)] is a rare Mendelian form of human hypertension, associated with hyperkalemia and hyperchloremic metabolic acidosis. In a subset of patients, the syndrome is due to mutations in the WNK1 and WNK4 genes encoding two serine-threonine kinases of the WNK [With No lysine (K)] family. The patients are particularly sensitive to thiazide diuretics, blockers of the NaCl cotransporter NCC. Accordingly, the analysis of a transgenic PHAII model (TgWNK4PHAII), overexpressing a mutated form of WNK4, revealed an increase in NCC expression...
and activity. However, others studies have shown that upregulation of NCC is not sufficient to induce PHAII. We have previously demonstrated the presence of an aniloride-resistant, thiazide-sensitive electroneutral NaCl absorption in the cortical collecting duct, resulting from the coupling of the Na⁺-driven Cl⁻/HCO₃⁻ exchanger (Ndcbe/Slc4a8) with the Cl⁻/HCO₃⁻ exchanger (pendrin/slc26a4) in the apical membrane of intercalated cells. Our purpose was to characterize the regulation of NaCl absorption by WNK4 in the collecting duct and its contribution to the PHAII phenotype.

Material and Results: We first measured the transepithelial fluxes in isolated microperfused CCD and showed that NaCl absorption was strongly stimulated in TgWNK4PHAII mice and fully inhibited by luminal addition of thiazide (N = 5 tubules in each group). Accordingly, Ndcbe protein expression was upregulated in TgWNK4PHAII mice. Quantification of pendrin activity in microperfused CCDs evidenced an increase in TgWNK4PHAII mice compared to controls (2.9 × 10⁻³ ± 0.37 pH units/s, n = 4 tubules vs 5.2 × 10⁻³ ± 0.64 pH units/s, n = 7 tubules, p < 0.05). We also observed an increase in the number of pendrin-positive intercalated cells in the CCD of transgenic mice (37.13 ± 3.64%, n = 1867 ICs from four control animals vs 49.68% ± 2.01%, n = 1955 ICs from four mutant mice, p < 0.05). Fractional volume of CCDs was increased in TgWNK4PHAII mice.

Discussion: These results indicate that mutation in WNK4 was associated with an increase in activity and volume of the cortical collecting duct. Increased NaCl absorption in the cortical collecting duct of TgWNK4PHAII mice was due solely to the activation of the electroneutral thiazide-sensitive Ndcbe/pendrin NaCl transport system.

SW03.S12-37
Characterization of subpopulations of early endosome autoantigen 1 (EEA1)-positive vesicles in HeLa cells
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EEA1 is considered to work as a tether mediated the first step of homotypic fusion of early endosomes upon its recruitment onto endosomal membrane consequently to Rab5 activation. However in HeLa cells incubated under serum depletion conditions for 12 h we found significant number of EEA1-positive vesicles and negligible level of EEA1 staining in cytoplasm. The total pool of EEA1-positive vesicles can be divided into two subpopulations differ in size, localization and intensity of EEA1 signal. The first one is represented by bright and relatively big vesicles localized presumably in juxtanuclear region of the cell. Experiments with Nocodazole have demonstrated that this localization is maintained by microtubules. The second subpopulation is represented by small vesicles randomly distributed throughout the cytoplasm and poorly decorated with EEA1. These faded vesicles make about 70% of the total EEA1 structures. Further analysis has demonstrated that about 50% of Rab5-positive structures contain also EEA1, but there are Rab5-negative/EEA1-negative vesicles as well EEA1-positive/ Rab5-negative vesicles. We conclude that the presence of Rab5 on the membrane is non always necessary for its association with EEA1. Quantitative analysis of EEA1 populations behavior during EGF receptor endocytosis favors the involvement of bright EEA1-vesicle subpopulation in mediating endosomal fusions at early stages of endocytosis.

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SW03.S12-38
MCT2 in brain glucose sensing
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Hypothalamic neurons of the arcuate nucleus control food intake, releasing orexigenic and anorexigenic neuropeptides in response to changes in glucose concentration. Several studies have suggested that the glucose-sensing mechanism is governed by a metabolic interaction between neurons and glial cells via lactate flux through monocarboxylate transporters (MCTs). Hypothalamic glial cells (tanyocytes) release lactate through MCT1 and MCT4; however, similar analyses in neuroendocrine neurons have yet to be undertaken. Using primary rat hypothalamic cell cultures and fluorimetric assays, lactate incorporation was detected. Furthermore, the expression and function of MCT2 was demonstrated in the hypothalamic neuronal cell line, GT1-7, using kinetic and inhibition assays. Moreover, MCT2 expression and localization in the Sprague Dawley rat hypothalamus was analyzed using RT-PCR, in situ hybridization and Western blot analyses. Confocal immunohistochemistry analyses revealed MCT2 localization in neuronal but not glial cells. Moreover, MCT2 was localized to ~90% of orexigenic and ~60% of anorexigenic neurons as determined by immunolocalization analysis of AgRP and POMC with MCT2-positives neurons. Thus, MCT2 distribution suggests that hypothalamic neurons control food intake using lactate to reflect changes in glucose levels.

SW03.S12-39
Low intensity 70.6 and 73 GHz frequencies electromagnetic irradiation and different antibiotics effects on E. coli ions transports properties
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The depressive effects of low intensity coherent EMI of 70.6 and 73 GHz frequencies on E. coli growth and survival properties, which enhanced with ceftriaxone or kanamycin antibiotics have been reported previously. This could be due to the reorganizations in membrane and alterations inside of cell. Therefore, the study of EMI and antibiotics action on ions fluxes is important to understand the role of bacterial membrane. E. coli carried out H⁺ and K⁺ exchange at the presence of energy source (glucose) by secreting 2 H⁺ by the proton F0F1-ATPase and uptaking a K⁺ by TrkA system [1].

This study showed that E. coli H⁺ and K⁺ transport systems activities were depressed after irradiation, which was strengthened with DCCD and ceftriaxone and kanamycin. The overall picture was the same for two frequencies, with a little difference. The depression of H⁺ and K⁺ fluxes rate was maximally achieved with the frequency of 73 GHz. EMI strengthened the effect of DCCD and 73 GHz and had more influence on H⁺ efflux inhibition, whereas 70.6 GHz on K⁺ influx. Also, EMI strengthened the ceftriaxone and kanamycin depressive effects on the overall and DCCD-inhibited H⁺ and K⁺ fluxes. The
The results of E. coli H+ and K+ transport systems activities depression by irradiation and the irradiation effect on DCCD and antibiotics action indicated about the EMI and antibiotics causing primary changes in bacterial membrane and its ion transporting systems. Especially, the effectiveness of combined action of EMI and antibiotics can be useful in future applications to overcome bacterial resistance to antibiotics [1].

References

SW03.S13 Biochemistry of Stress Response (III-S13)

SW03.S13–1
Heat shock proteins can be targets of inflammation controlling regulatory T cells
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The fundamental problem in autoimmune diseases such as rheumatoid arthritis, type I diabetes, MS and inflammatory bowel diseases, is faulty regulation of the inflammatory process. It was once thought that the pathogenesis of autoimmune inflammation was initiated by the accidental emergence of a forbidden clone of self-reactive effector T cells; however, it is now clear that the immune systems of healthy people are populated with T cells and B cells bearing receptors that can bind self-antigens. Inflammatory disease results from the failure of the immune system to regulate its own potentially dangerous cells. Thus, the rational goal of therapy in diseases of unregulated inflammatory activation is to reinstate physiological regulation. Indiscriminate suppression of immune cells and molecules is costly in side effects and does not provide adequate therapy of the basic condition.

Re-establishing physiological self-tolerance in autoimmunity is thought to depend on self-reactive regulatory T cells (Tregs). Exploiting these antigen-specific regulators is hampered by the obscure nature of disease-relevant auto-antigens. Since the initial discoveries of the protective role of HSP60 in arthritis of rodents (1) and humans (2), T cell recognition of endogenous HSP60, over-expressed due to local inflammatory stress, was found to be one of the possible underlying mechanisms (3). With the re-invention of Tregs as the main tolerance promoting cells, antigen specific activities of Tregs have become an expanding area of interest (4). Recently we have uncovered potent disease-suppressive Tregs recognizing Heat shock protein (Hsp) 70 self-antigens, enabling selection of Tregs as the main tolerance promoting cells, antigen specific activities of Tregs have become an expanding area of interest (4). Recently we have uncovered potent disease-suppressive Tregs recognizing Heat shock protein (Hsp) 70 self-antigens, enabling selecting activity in inflamed tissues (6). Hsp70 turned out to be a major contributor to the MHC Class II ligandome and we have shown that a conserved Hsp70-epitope (B29) is abundantly present in murine MHC Class II. Upon transfer, B29-induced CD4+CD25+Foxp3+ T cells suppressed established proteoglycan-induced arthritis (PGIA) in mice. These self antigen-specific Tregs were activated in vivo and when using Lymphocyte Activation Gene-3 (LAG-3) as a selection marker, as little as 4,000 cells sufficed to fully inhibit arthritis. Furthermore, depletion of transferred Tregs abrogated disease suppression. Transferred cells exhibited a stable phenotype and were found in joints and draining lymph nodes up to 2 months after transfer. Given that B29 can be presented by most human MHC molecules and B29 inhibited arthritis in HLA-DQ8 transgenic mice, we feel that therapeutic vaccination with selected HSP peptides can be an effective route for induction of anti-inflammatory Tregs as a novel intervention for chronic inflammatory diseases (5).

References

SW03.S13–2
Control of cancer cell survival by heat shock protein 70 and sphingomyelin metabolism
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Transformation is associated with a decreased stability of lysosomal membranes and an enhanced sensitivity to lysosomal cell death pathways induced by various anti-cancer drugs. This sensitization is at least partially brought about by the increased cysteine cathepsin expression and activity and cathepsin-mediated degradation of lysosomal membrane stabilizing proteins LAMP-1 and LAMP-2. On the other hand, lysosomal heat shock protein 70 (Hsp70) promotes the survival of cancer cells by enhancing the activity of lysosomal acid sphingomyelinase (ASM) thereby stabilizing the lysosomal membranes. These data prompted us to investigate whether lysosomal ASM could serve as a direct target for cancer therapy. Data presented here show that ASM activity is essential for the lysosomal stability and survival of transformed cells. Importantly, ASM can be inhibited by an experimental anti-cancer agent siamesine as well as by several widely used and relatively safe cationic amphiphilic drugs (tricyclic antidepressants, antihistamines and calcium channel blockers) that trigger lysosomal cancer cell death even in apoptosis- and multidrug-resistant cells. The striking cancer selectivity of ASM inhibitors is associated with significantly reduced expression of the ASM encoding sphingomyelin phosphodiesterase 1 (Smpd1/SMPD1) gene in transformed cells and various human cancers. Thus, ASM inhibitors should prove efficacious in tumors with low sphingomyelinase activity, or when combined with classic chemotherapy, even to treat tumors that have acquired therapy resistance.
SW03.S13–3

Autophagy and neurodegeneration

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Intracellular protein aggregation is a feature of many late-onset neurodegenerative diseases, including Parkinson’s disease, tauopathies, and polyglutamine expansion diseases such as Huntington’s disease (HD). Many of these mutant proteins, like those causing HD, cause disease via toxic gain-of-function mechanisms. Therefore, the factors regulating their clearance are crucial for understanding disease pathogenesis and for developing rational therapeutic strategies.

The two major intracellular protein degradation pathways are the ubiquitin-proteasome system and (macro)autophagy. Autophagy is initiated by double-membraned structures, which engulf portions of cytoplasm. The resulting autophagosomes ultimately fuse with lysosomes, where their contents are degraded. I will describe our recent studies that implicate the plasma membrane as a source for autophagosomes, before focussing on the roles of autophagy in neurodegeneration.

We showed that the autophagy inducer, rapamycin, reduced the levels of mutant huntingtin and attenuated its toxicity in cells, and in Drosophila and mouse HD models. We have extended the range of intracellular proteinopathy substrates that are cleared by autophagy to other related neurodegenerative disease targets and have provided proof-of-principle in cells, Drosophila and mice. In order to induce autophagy long-term, we have been striving to identify safer alternatives to the mTOR inhibitor, rapamycin. To this end, we have been trying to discover novel components of the autophagy machinery and new signalling pathways and drugs that impact on autophagy. While autophagy induction is protective in models of various neurodegenerative diseases, certain other conditions, including lysosomal storage disorders, are associated with compromised autophagy. I will review these data and then describe how impaired autophagy compromises cellular processes, including the ubiquitin-proteasome system.

SW03.S13–4

Heat shock protein 70 based anti-tumor therapies

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Immunization of mice with a 14-mer peptide TKDNNLLGR-FELSG, termed ‘TKD’, comprising amino acids 450–461 (aa450-461) in the C-terminus of inducible Hsp70, resulted in the generation of an IgG1 mouse monoclonal antibody (mAb) cmHsp70.1 (Stangl et al. 2011). The epitope recognized by cmHsp70.1 mAb, is frequently detectable on the cell surface of human and mouse tumors but not on normal tissues.

Intraoperative imaging as well as FMT analysis results revealed that fluorophor and radiolabelled cmHsp70.1 mAb specifically detects tumors in mice and does not cross-react with normal tissues.

Following ionizing irradiation or chemotherapy the expression density of Hsp70 is significantly enhanced selectively on tumor cells. Therefore, membrane Hsp70 might serve as a tumor-specific target structure for immune-based therapies and monitoring of clinical outcome. In preclinical models we have shown that the cmHsp70.1 mAb induces antibody dependent cellular cytotoxicity (ADCC) of membrane Hsp70 positive mouse tumors. Three consecutive i.v. injections of the cmHsp70.1 mAb into mice bearing CT26 tumors significantly inhibited tumor growth and enhanced the overall survival. These effects were associated with infiltrations of NK cells, macrophages and granulocytes.

NK cells that had been stimulated with Hsp70 peptide plus low dose IL-2 have been found to kill Hsp70 membrane-positive tumors but not their Hsp70 membrane-negative counterparts via granzyme B mediated lysis. In a phase I clinical trial feasibility, safety, and tolerability of Hsp70 peptide plus IL-2 stimulated, autologous NK cells has been demonstrated. Presently, a phase II proof-of-concept trial with ex vivo TKD/IL-2 stimulated autologous NK cells of non-small cell lung carcinoma (NSCLC stage IIa/IIb) patients after radiochemotherapy is ongoing.

In the absence of perforin, granzyme B can enter tumor cells via endocytosis of membrane Hsp70. We established a mammalian (HEK293 cells) expression system to produce high yields of human granzyme B. First results in a xenograft tumor mouse model reveal tumor shrinkage in immunodeficient mice after i.v. injections of granzyme B (Gehrmann et al. 2012).

References


SW03.S13–5

Role of heat shock proteins in cancer initiation and progression

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Heat shock proteins are often expressed at elevated levels in many cancers. Their expression correlates with tumor progression, suggesting that they play a special role in cancer. Indeed, using transgenic animals we have found that the major heat shock protein Hsp72 is essential for tumorigenesis induced by the breast cancer oncogene Her2. In cancer initiation, Hsp72 was needed to suppress oncogene-induced senescence (OIS). In addition to OIS, Hsp72 regulates a number of other pathways that control various stages of tumor development, including cancer cell migration and invasion, angiogenesis and metastases.

The major concept in the field is that cancer cells have special requirements for Hsp72 and other chaperones because they experience the pressure of proteotoxic stress. We, however, found that such pressure is minimal, and that in addition to its chaperone function, Hsp72 plays a purely signaling role via interaction with its co-factor BAG3. Indeed, disruption of this interaction mimics effects of Hsp72 depletion on cancer signaling pathways. We have developed a small molecule that disrupts interaction between Hsp72 and BAG3, and found that it has similar effects. This molecule can be used as lead for anti-cancer drug design.

SW03.S13–6

Small Hsps phospho-oligomeric structure dependent interactome as therapeutic target

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Among the different human small Hsps, HspB1 (Hsp27) is characterized by its dynamic phosphorylation and heterogeneous oligomerization in response to changes in cell physiology. The phenomenon is particularly intense and specific when cells are...
exposed to different death inducers. The structural organization of HspB1 acts as a sensor which, through reversible modifications, allows cells to adapt and/or mount a protective response. A large number of HspB1 interacting client partners have already been identified. Specific changes in oligomer-phosphorylation organization may therefore allow HspB1 to interact with the more appropriate polypeptides and to subsequently modulate their folding/activity and/or half-life. In cancer, HspB1 is tumorigenic, stimulates metastasis and provide cancer cells with resistance to many anti-cancer drugs, so compounds aimed at disrupting HspB1 interaction with deleterious pro-cancer protein substrates could be promising anti-cancer drugs. AlphaB-crystallin (HspB5) as well as HspB8 have also been shown to interact with many different polypeptides. This could indirectly link these small Hsps to multiple cellular functions and explain the apparently unrelated effects associated to their over- or under-expression. Hence, developing small Hsps structure-based interfering strategies could lead to the discovery of new therapeutic drugs that can either inhibit or stimulate the function of client polypeptides involved in pathological diseases.

SW03.S13–8
Hsp70 and its co-chaperones in normal and pathologic CNS
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Molecular chaperones Hsp70 and its co-chaperones possess neuroprotective activity that is demonstrated in cell and animal models. In pathologies associated with the accumulation of proteins with abnormally long polyglutamine tracts, this activity is linked to the ability of Hsp70 to prevent the formation of aggregates by binding the latter components. Hsp70 when being over-expressed in cells with growing mutant huntingtin aggregates was found to bind both polyglutamine tracts and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and to inhibit their complex as well as aggregate progression. Thus we proved that Hsp70 in pathologies associated with aggregation of mutant proteins has besides the latter other targets which are normal proteins with a tendency to aggregate. To check whether Hsp70 possesses protective activity in another model of CNS pathology, drug-induced epilepsy, we employed technology of the chaperone delivery directly to brain of rats preliminarily treated with seizure inducers. In this study Hsp70 was shown to penetrate within specialized sections of brain and to reduce both the level of specific neuronal activity and the period of seizure activation. These data together with our earlier results prove the relevance of Hsp70 administered directly in brain as an efficient therapeutic tool. J-domain-containing proteins, Hdj1/Hsp40, Hdj2 and other, are known to assist Hsp70 in normal and stressed cells; this property persuaded us to explore whether this kind of assistance extends to the role of Hsp70 that it plays in normal brain physiology, particularly slow-wave sleep. We used lentivirus construct with anti-Hdj1 shRNA to reduce the co-chaperone content in hypothalamus and found that that the diminishing Hdj1 content caused the considerable increase of slow-wave sleep and the reduction of anxiety level. Taken together these data demonstrate high therapeutic potential of molecular chaperones of Hsp70 and DnaJ family proteins in treatment quite different neurological disorders.
Their subcellular localisation was compared and ER mobility assessed by fluorescence recovery after photobleaching (FRAP). The WT-C185A-GFP and WT-C185S-GFP mutants were indistinguishable from WT-GFP rod opsin in levels of expression and glycosylation patterns but appeared to traffic to the plasma membrane faster than WT rod opsin. Furthermore, P23H-C185A-GFP and P23H-C185S-GFP were retained in the ER, like P23H-GFP, but had greater mobility in the ER than P23H-GFP rod opsin. Overall, these mutants support a role for disulphide bond formation/reduction in rhodopsin biogenesis and disease and provide a new insight into a potential role of the C185 residue in opsin biogenesis.

SW03.S13–12
Transcriptional changes in rhodopsin Retinitis Pigmentosa
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Retinitis Pigmentosa (RP) is a group of inherited retinal diseases characterized by progressive degeneration of rod and cone photoreceptors in the retina. Mutations in rhodopsin are the most common cause of autosomal dominant RP (ADRP) and the Pro23His (P23H) mutation is the most commonly described gene defect for RP in North America. Transgenic animal models have been developed, however, the exact mechanism by which the P23H mutation leads to photoreceptor degeneration is still uncertain. The aim of this study was to investigate changes in gene transcription in mouse models of P23H ADRP. MouseWG-6 v2 (Illumina) microarrays were used to compare the retinal transcriptome of VPG transgenic and P23H knock-in mice with age matched controls early in disease and close to the peak of photoreceptor cell death. The analysed expression profiles suggest a genome-wide response to retinal degeneration with a number of genes upregulated and a substantial overlap between the two models. Among the induced transcripts we observed mRNA belonging to the Janus kinase/Signal Transducer and Activator of Transcription (Jak/STAT) signaling pathway, Muller cell activation markers, and protective cytokines. Importantly, Edn2 was the most upregulated transcript in both mouse models. Interestingly, a similar profile of transcript induction has been previously reported in other models of inherited or experimental retina degeneration. These data support the hypothesis that diverse types of photoreceptor damage activate a common intraretinal signaling pathway, which works as an endogenous response mechanism to retinal injury.

SW03.S13–13
Lysosomal rerouting of Hsp70 trafficking as a potential immune activating tool for targeting melanoma
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Tumor specific cell surface localization and release of the stress inducible heat shock protein 70 (Hsp70) stimulate the immune system against cancer cells. A key immune stimulatory function of tumor-derived Hsp70 has been exemplified with the murine melanoma cell model, B16 overexpressing exogenous Hsp70. Here we investigated the mechanism of Hsp70 transport to the
surface and release in melanomas with low and high levels of Hsp70. Cellular fractionation and imaging data showed that Hsp70 is present in the endolysosomal system, and excess Hsp70 accumulates in the lysosomes. In cells with low level of Hsp70, tested in flow cytometry measurements, transport to the surface and endocytosis were confined to the endosomes. In contrast, excess Hsp70 triggered a switch of Hsp70 trafficking from endosomes to lysosomes as determined from biochemical, fluorescence quenching and imaging data. Lysosomal rerouting of Hsp70 trafficking resulted in an elevated concentration of surface Hsp70 shown in AFM recognition images, and enabled active release of Hsp70 measured with ELISA. In fact, hyperthermia triggered immediate active lysosomal release of soluble Hsp70 from cells with excess Hsp70. Furthermore, excess Hsp70 enabled lysosomal targeting and subsequent release of internalized surface Hsp70, where vesicular fusion events were visualized in dual color TIRF measurements. Finally, active release of the immune stimulatory Hsp70 and surface Hsp70 was found to require acute excess of Hsp70. Altogether, we show that excess Hsp70 expressed in B16 melanoma cells diverts Hsp70 trafficking from endosomes to lysosomes, thereby supporting its surface localization and lysosomal release. An acute excess-induced lysosomal rerouting and secretion of Hsp70 is proposed as a promising tool to stimulate anti-tumor immunity targeting melanoma.

**SW03.S13–14**

*Exogenous heat shock protein Hsp70 cycling through cancer cells can enhance anti-tumor innate and adaptive immune response*

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Heat shock protein Hsp70 is found in the extracellular milieu and could exhibit stimulatory activity on various immunocompetent cells. Though several hypothesis explaining chaperone immunomodulatory activity were proposed still the work remains to be performed that will elucidate the exact mechanism of exogenous Hsp70 function. In our study we analyzed the interaction of Hsp70 with tumor cells and the influence of this cooperation on the host's immune system in vivo. Exogenously delivered in vitro Hsp70 could penetrate tumor cells (C6 glioma, B16 melanoma, K562 erythroleukemic and U937 cells) and induce the translocation of intracellular Hsp70 to the plasma membrane as was demonstrated by confocal microscopy, flowcytometry. This increased the sensitivity of cancer cells towards lytic activity of NK – cells in cytotoxicity splenocyte test (CTL-test) up to 35–40% (p < 0.001). Furthermore, exogenous Hsp70 induced the export of intracellular chaperone (probably with tumor peptides) into extracellular space thus contributing to the enhancing of the adaptive immune response. In the model of glioma C6 in rats locally delivered Hsp70 accumulated inside tumor cells and caused the delay in tumor growth according to magnetic resonance imaging (MRI). Temporal delay in tumor progression resulted in the increased survival of tumor-bearing animals. Intratumorally injected Hsp70 enhanced systemic anti-tumor response in the CTL-test. Elevated activity of splenocytes corresponded to the 10-fold increase of the induced production of INF-γ in the ELISA assay. The immunofluorescence analysis of brain tumor showed the massive infiltration by T-lymphocytes (CD3+, CD4+, CD8+) and NK-cells (CD56+). Based on our in vitro and in vivo data we proposed a novel concept that clarifies the anti-cancer immunomodulatory activity of chaperone Hsp70.

**SW03.S13–15**

*Tumor-derived exosomes with extramembrane HSP70: cancer therapeutic target?*

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Cancer cells secrete nanovesicles called exosomes, which express the membrane stress protein: HSP70. HSP70 can activate myeloid derived suppressor cells (MDSC), which have an immuno-suppressive activity, via an interaction with Toll-like receptor 2 (TLR2) present on their surface. This activation results in the induction of JAK/STAT3 pathway. In this context, we investigated in vitro and in vivo the HSP70 inhibitor peptide’s effect on the anti-tumor response. Our results show that this inhibitor can block the interaction between HSP70 and TLR2 and consequently the activation of MDSC. In vivo, injection of the inhibitor favors the anti-tumor immune response and potentiates the anti-cancer effect of chemotherapeutic agents, such as cisplatin.

Preliminary results also showed the presence of human tumor exosomes in urine samples. Detection of HSP70 on these exosomes could serve in early diagnosis and / or in monitoring treatment patients. Ultimately, this work could allow the development of more effective anti-cancer therapies in combination with HSP70 inhibitors.

**SW03.S13–16**

*Role of aquaporin isoforms on Nox-dependent redox signalling involved in proliferation of leukaemia cells*

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NAD(P)H oxidases (Nox) are a family of membrane-associated enzymatic complexes that, transferring electrons from NAD(P)H inside the cell to molecular oxygen across the membrane, generate superoxide anion (O2·−) and/or hydrogen peroxide (H2O2) outside the cell [1]. Nox-produced ROS can act as messengers in redox signalling and hydrogen peroxide is considered the main effector due to its relative stability. Although it is now confirmed the involvement of ROS in biosignalling, it still remains unclear whether the ROS generated extracellularly by Nox can permeate through the plasma membrane by simple diffusion or mediated by transport proteins. Recent experimental evidence suggest that two isoforms of aquaporins (AQP3 and AQP8) possess the ability to channel H2O2 across the membranes and that they may have an important role in redox signalling [2]. Our attention has been focused on ROS involved in signalling transduction pathways contributing to cancer development. In particular, we have previously demonstrated that Nox-generated ROS sustain glucose uptake and cellular proliferation in leukaemia cell lines [3].

In this study, we assess whether specific aquaporin isoforms play a role in facilitating Nox-produced H2O2 transport through the plasma membrane affecting downstream pathways linked to cell proliferation in leukaemia cells. We demonstrated that the treatment with AgNO3, a potent aquaporin inhibitor, causes a decrease in intracellular ROS level both when H2O2 is produced...
by Nox enzymes and when it is added exogenously to the medium; on the contrary overexpression of AQP8 and AQP3 causes an increase in the intracellular ROS level when hydrogen peroxide is added. Furthermore, aquaporin inhibition causes a significant decrease in glucose transport in HL60 and B1647 cells and VEGF-induced intracellular ROS accumulation is augmented when AQP3 or AQP8 are overexpressed. In conclusion, our data suggest that AQP3 and AQP8, but not AQP1, are able to transport Nox-generated H₂O₂ across the plasma membrane supporting leukemia cell proliferation.

References

SW03.S13–17
The small heat shock proteins: important players in small packages
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The occurrence of protein misfolding diseases indicates that, under certain conditions, the protein quality control systems of cells (i.e. those involved in proteostasis) can be ‘overwhelmed’, enabling protein aggregates to form. The small heat-shock molecular chaperone proteins (sHsps) are one of the cell’s first-lines of defence against protein aggregation [1]. Most models of sHsp chaperone action suggest that they act as holdase chaperones, i.e. they bind to and stabilise partially folded (intermediate) states of proteins, to prevent their aggregation. Our work seeks to further explore the mechanisms used by sHsps to prevent protein aggregation. Here we present recent work that shows that sHsps can interact with aggregating proteins at multiple stages along the aggregation pathway. However, the description of sHsps as ‘holdase’ chaperones does not fully encompass their chaperone activities. Using alpha-lactalbumin as a model aggregation prone protein we have found that the sHsp, alphaB-crystallin, can interact transiently with target proteins to prevent their aggregation [2]. Moreover, alphaB-crystallin binds directly to amyloid fibrils and, in doing so, acts to stabilise them and decreases their toxicity to cells. Thus, by binding to fibrils, sHsps may have a second protective role in preventing the toxicity associated with protein misfolding. Based on our findings we propose a revised model of sHsp chaperone action in which the the conformational stability of the protein intermediate, which is a precursor to aggregation, acts transiently with target proteins to prevent their aggregation from further aggregation. Using alpha-lactalbumin as a model aggregation prone protein we have found that the sHsp, alphaB-crystallin, can interact transiently with target proteins to prevent their aggregation [2]. Moreover, alphaB-crystallin binds directly to amyloid fibrils and, in doing so, acts to stabilise them and decreases their toxicity to cells. Thus, by binding to fibrils, sHsps may have a second protective role in preventing the toxicity associated with protein misfolding. Based on our findings we propose a revised model of sHsp chaperone action in which the the conformational stability of the protein intermediate, which is a precursor to aggregation, acts transiently with target proteins to prevent their aggregation.

References

SW03.S13–18
The role of structure and regulation of hsp70 gene family in the adaptation to extreme conditions
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The heat-shock response is a universal defensive mechanism described in all groups of living organisms. This response is characterized by the synthesis of a small group of conserved proteins, designated ‘heat shock proteins’ (Hsps). The heat shock proteins belonging to the Hsp70 family play a crucial role in the protection of cells due to their chaperoning functions. It is well known, that induction and accumulation of inducible form of Hsp70 (Hsp70i) after mild heat treatment promotes tolerance to the subsequent more extreme temperature. The phenomenon termed ‘induced thermotolerance’ [1]. In our study we explored D. melanogaster strain lacking all hsp70i genes that were deleted by genetic manipulations [2]. We also cloned and sequenced hsp70i genes from another Diptera species (Stratommys singularior) that inhabits warm highly mineralized lakes. The latter species is characterized by a high constitutive level of Hsp70 and extraordinary high thermotolerance [3]. We transformed D. melanogaster strain lacking all hsp70i genes with a iHsp70 copy isolated from S. singularior.

Surprisingly, our analysis demonstrates, that the promoter of hsp70i gene of S. singularior is not able to induce transcription of correspondent mRNA in the cells of D. melanogaster under heat shock conditions, although the promoter seems to contain all major components necessary for induction by HS. In the other series of transformation experiments we used the D. melanogaster own hsp70i promoter and were able to induce expression of S. singularior hsp70i genes in the genome of D. melanogaster. We also demonstrated that S. singularior Hsp70 effectively provides thermotolerance when expressed in the foreign (D. melanogaster) host species. The accumulated data allow to postulate that fine tuning of hsp70i genes is necessary for adaptation of species to various environmental conditions and may quickly diverge even within one animal order (Diptera).

References

SW03.S13–19
Effect of endemic plants extracts on biological macromolecules
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Reactive oxygen species (ROS) are generated during normal aerobic metabolism, but they may be toxic in higher concentrations causing a great damage of macromolecules such as DNA, proteins and lipids. Oxidative stress which usually generates increased amount of ROS play an important role in an etiology and pathogenesis of various diseases such as cardiovascular disease, cancer, diabetes and neurodegenerative disease. Cells possess mechanisms for radical detoxification that are not full effective in case of drastic stress conditions. Some compounds with important antioxidant activity originated in food and dietary supplements may have protective effect on ROS caused damages. Thus, numerous epidemiological and clinical studies support hypothesis that regular consumption of products reach effective in case of drastic stress conditions. Some compounds with important antioxidant activity originated in food and dietary supplements may have protective effect on ROS caused damages. Thus, numerous epidemiological and clinical studies support hypothesis that regular consumption of products reach
Griseb, Micromeria croatica (Pers.) Schott and Rhamnus intermediens Steud. et Hochst) against ROS damaging of important biological macromolecules: lipids, proteins and DNA. Content of polyphenolic compounds (total phenol, flavonoids, hydroxyecynamic acids, proanthocyanidins and individual phenolic acids) was determined in plant extracts using spectrophotometer and UPLC-MS/MS. Furthermore, in vitro antioxidant activities of plant methanol extracts were screened using DPPH, ABTS and FRAP methods. All four tested extracts shown high in vitro antioxidant capacity and DNA protection effects. However, their effects on protein and lipid protection were diverse and will be discussed.

SW03.S13–20
New insights into deleterious impacts of in vivo glycation on albumin antioxidant activities
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Albumin constitutes the most abundant circulating antioxidant and prevents oxidative damages. However, in diabetes, this plasmatic protein is exposed to several oxidative modifications, which impact on albumin antioxidant properties. Most studies dealing on albumin antioxidant activities were conducted on in vitro modified protein. Here we tried to decipher whether reduced antioxidant properties of albumin could be evidenced in vivo. For this, we compared the antioxidant properties of albumin purified from diabetic patients to in vitro models of glycated albumin. Both in vivo and in vitro glycated albumins displayed impaired antioxidant activities in the free radical-induced hemolysis test. Surprisingly, the ORAC method (Oxygen Radical Antioxidant Capacity) showed an enhanced antioxidant activity for glycated albumin. Faced with this paradox, we investigated antioxidant and anti-inflammatory activities of our albumin preparations on cultured cells (macrophages and adipocytes). Reduced cellular metabolism and enhanced intracellular oxidative stress were measured in cells treated with albumin from diabetics. NF-kB–mediated gene induction was higher in macrophages treated with both type of glycated albumin compared with cells treated with native albumin. Anti inflammatory activity of native albumin is significantly impaired after in vitro glycation and albumin purified from diabetics significantly increased IL6 secretion by adipocytes. Expression of receptor for advanced glycation products is significantly enhanced in glycated albumin-treated cells. Our results bring new evidences on the deleterious impairments of albumin important functions after glycation and emphasize the importance of in vivo model of glycation in studies relied to diabetes pathology.

SW03.S13–21
Role of ROS in normalization of primary cilia length altered by ischemia/reperfusion insult
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The primary cilium is a microtubule-based non-motile organelle that extends from the surface of most mammalian cells, including renal tubule epithelial cells. Recent studies suggest that the primary cilium is associated with kidney diseases including acute kidney injury. Here, we investigated the alteration of primary cilium length during epithelial cell injury and repair, following an ischemia/reperfusion (I/R) insult, and the role of reactive oxygen species in this alteration. Thirty minutes of bilateral renal ischemia induced severe renal tubular cell damage and an increase of plasma creatinine (PCr) concentration. Between 8 and 16 days following the ischemia, the increased PCr returned to normal range, though without complete histological restoration. Compared with the primary cilium length in normal kidney tubule cells, the primary cilium length was shortened 1 day following ischemia, increased over normal 8 days after ischemia, and then returned to near normal 16 days following ischemia. The primary cilium length of proliferating cells, as determined by 5′-bromo-2′-deoxyuridine (Brdu) incorporation, was shorter that in non-Brdu incorporating cells. Mature renal tubule cells had shorter primary cilia than differentiating cells. Superoxide and lipid peroxidation levels in the kidneys following I/R were greater than in sham-operated mouse kidneys. Treatment with Mn(III) Tetrakis(1-methyl-4-pyridyl) porphyrin, a superoxide dismutase (SOD) mimetic antioxidant, during the recovery of damaged kidneys, from 2 days after ischemia to 8 days, accelerated normalization of primary cilium length concomitant with a decrease of superoxide and lipid peroxidation levels, and morphological recovery in the kidney. Taken together, our results demonstrate that primary cilium length is shortest in injured and proliferating cells, longest in differentiating cells, and intermediate in mature cells, suggesting that the length of primary cilium may be a marker of cell fate. In addition, the length of primary cilium appears to be regulated, at least in part, by reactive oxygen species.

SW03.S13–22
Stimulation of mitochondrial biogenesis and mitochondrial elongation during preconditioning: the brain adapting to survive!
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Brief episodes of sublethal hypoxia reprogram brain response to face subsequent lethal stimuli by triggering adaptive and pro-survival mechanisms – a phenomenon denominated by hypoxic preconditioning (HP). Notably, we previously demonstrated the effectiveness of HP in preventing Alzheimer’s disease-related pathological features including mitochondrial dysfunction. Given the importance of mitochondria in determining cells fate, the present study was devoted to monitor the structural and functional alterations of brain mitochondria in response to a well-established protocol of HP induced by the cyclic exposure to moderate hypoxia (10% O2, during 2 h) with intervening 24 h reoxygenation periods, during three consecutive days. Several parameters related with mitochondrial bioenergetic function, biogenesis, and fusion and fission machinery were evaluated in the cortex and hippocampus of adult rats immediately, 6 and 24 h after the last hypoxic session. HP induced a decrease in respiratory state 2 and an increase in ADP/O ratio in brain cortical and hippocampal mitochondria. Immediately after the last hypoxic episode, a significant increase in the protein levels of nuclear respiratory factor-1, and mitochondrial transcription factor A was observed. Twenty-four hours after the last hypoxic episode, a shift in the mitochondrial fusion-fission balance towards fusion occurred, as evidenced by the significant increase in the protein levels of optineurin protein 1 and a decrease in the protein levels of dynamin-like protein 1 in the brain cortex, and the significant reduction in the fission protein 1 levels in the hippocampus. Consistently, the electron microscopy analysis revealed that HP generated mitochondria with an elongated phenotype. Overall, these results indicate that HP enhances mitochondrial bioenergetic function, probably due to a coordinated
interplay between mitochondrial biogenesis and fusion/fission events, increasing brain tolerance.

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SW03.S13–23
Ursolic acid sensitizes prostate cancer cells to TRAIL-mediated apoptosis
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Prostate cancer is one of the most commonly occurring malignancies in men, and because existing treatments are not able to manage this neoplasm adequately, novel approaches are needed. Although TRAIL has strong antitumor activity via the induction of apoptotic cell death in a wide range of tumor cell types and has negligible toxicity to most normal cells, some prostate carcinoma cells are resistant to the apoptotic effects of TRAIL. Therefore, combinatorial approaches with TRAIL and different chemotherapeutic agents have been developed to overcome the resistance of cancer cells to TRAIL. Here, we investigated the sensitizing effects of ursolic acid (UA), a pentacyclic triterpenoid found in many plants, on TRAIL-induced prostate cancer cell apoptosis. We found TRAIL-induced prostate cancer cells apoptosis was significantly enhanced by UA, and UA induced CHOP-dependent DR5 up-regulation. This study shows the use of UA as a sensitizer for TRAIL-induced apoptotic cell death offers a promising means of enhancing the efficacy of TRAIL-based prostate cancer treatments.

SW03.S13–24
Role of thioredoxin, thioredoxin reductase and peroxiredoxin in redox-dependent mechanism of development of multidrug resistance in cancer cells
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Introduction: Redox-dependent processes to a significant extent determine functional actions of many proteins and as a consequence redox-dependent regulation of important cellular functions such as proliferation, differentiation, apoptosis. The aim of the present study was the investigation of the role of thioredoxin, thioredoxin reductase and peroxiredoxin in development of cancer cells resistance to anticancer agent doxorubicin (DOX) possessed pro-oxidant action.

Methods: mRNA of genes encoding isoforms of thioredoxin – Trx1, Trx2, isoforms of thioredoxin reductase –TrxR1, TrxR2 and isoforms of peroxiredoxin – Prx1, Prx5, Prx6 was determined using qRT-PCR. Level of Nrf2 was measured by Western blotting.

Results: It was found that the development of resistance to DOX in K562, MCF-7 and SKOV-3 cells was accompanied by increased expression of PRDX6 gene. Enhancement of PRDX1 and PRDX2 gene expression was obtained in SKVLB cells. Growth of expression of TRX2 and TRXR2 genes was found in resistant K562/DOX, MCF-7/DOX, SKVLB cells whereas a significant increase in the expression of TRX1 gene was detected only in SKVLB cells. Elevated level of Nrf2 transcriptional factor was observed in all three types of resistant cancer cells.

Discussion: The growth of expression of genes controlled Trx/Prx system may be estimated as the important part of adaptive antioxidant response in redox-dependent mechanism of development of the cancer cell resistance to DOX which has cell type-specific character.

SW03.S13–25
Antiproliferative and anticarcinogenic effects of an aqueous preparation of Urtica urens in human hepatocarcinoma HepG2 cells
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Despite recent advances in early diagnosis and treatment, cancer is still the first leading cause of death during the last 20 years. In addition to conventional treatments such as chemotherapy and radiation therapy alternative and complementary therapies are also frequently used among the cancer patients not only in Turkey but also all over the world. Urtica urens L. (Small Stinging Nettle), belonging to the Urticaceae family’s genus Urtica, is the most commonly used plant in alternative and complementary treatment of cancer patients in Turkey. However, studies in literature showed that there are limited studies investigating the anti-carcinogenic activity of this plant. Therefore, the present study was aimed to investigate the anti-carcinogenic effect of small nettle leaves water extract in human liver cancer cell line. In this respect, water extract was applied in different concentration to the HepG2 (human hepatocarcinoma cell line) cells (1 × 10³ cells/well) for 48 h. At the end of 48 h, the survival rate of the cell was measured by WST-1 at 450–690 nm. EC50 was found to be 107.8 mg/ml extract for HepG2 cells. This dose was selected for further studies. Moreover, qRT-PCR results showed that the mRNA level of apoptotic protein Bax was increased 115% by Urtica urens’ water extract. Also, mRNA levels of tumor suppressor proteins namely p53 and PTEN were increased 152% and 373%, respectively (p < 0.05). Moreover, extract treatment caused 95% and 125% in CDKN1A and CDKN2A that are involved in cell cycle regulation. However, mRNA levels of other cell cycle regulator proteins, cyclin dependent kinase-2 and 4, were decreased 35% and 29% in extract treated cells, respectively.

In conclusion, present study demonstrated that Urtica urens employ the anti-carcinogenic effect by stimulating different pathways including apoptosis, cell cycle regulation and tumor suppression. Moreover, these results suggest the hypothesis that the small nettle contains promising phytochemicals that may be used in cancer treatment. Despite recent advances in early diagnosis and treatment, cancer is still the first leading cause of death during the last 20 years. In addition to conventional treatments such as chemotherapy and radiation therapy alternative and complementary therapies are also frequently used among the cancer patients not only in Turkey but also all over the world. Urtica urens L. (Small Stinging Nettle), belonging to the Urticaceae family’s genus Urtica, is the most commonly used plant in alternative and complementary treatment of cancer patients in Turkey. However, studies in literature showed that there are limited studies investigating the anti-carcinogenic activity of this plant. Therefore, the present study was aimed to investigate the anti-carcinogenic effect of small nettle leaves water extract in human liver cancer cell line. In this respect, water extract was applied in different concentration to the HepG2 (human hepatocarcinoma cell line) cells (1 × 10³ cells/well) for 48 h. At the end of 48 h, the survival rate of the cell was measured by WST-1 at 450–690 nm. EC50 was found to be 107.8 mg/ml extract for HepG2
toward rational therapeutics of neurodegenerative diseases.

Together, we proposed the interplay among GSKIP, PKA, fission and conferring neuroprotection by H2O2 stress. Taken AKAP to promote PKA to phosphorylate Drp1 at Ser637 rather protection under H2O2 insult. Finally, to convergent the evidences of suggested that PKA binding domain is associated with neuropro-

oxidative stress, which cleaved caspase-3/-7 and PARP expression apoptotic death than GKSIP L130P group under H2O2 induced-

overexpressed GSKIP wt and V41/L45P, but not L130P, and sufficient to regulate neurite outgrowth in SH-SY5Y cells. Our data showed that both GFP-GSKIP wt and GFP-GSKIP V41/L45P could inhibit the differentiation with retinoic acid. Our data showed that both GFP-GSKIP wt and GFP-GSKIP V41/L45P could inhibit the neurite outgrowth, suggesting that GSK3beta biding is required and sufficient to regulate neurite outgrowth in SH-SY5Y cells. Further, overexpressed GSKIP wt and V41/L45P, but not L130P, increased Ser9-phosphorylated GSK3beta protein levels in SH-SY5Y under H2O2 induced-oxidative stress. Furthermore, cells overexpressing GSKIP V41/L45P were observed for a higher apoptotic death than GKSIP L130P group under H2O2 induced-oxidative stress, which cleaved caspase-3/-7 and PARP expression were enhanced. In addition to Gsk3 function, our data further suggested that PKA binding domain is associated with neuroprotection under H2O2 insult. Finally, to convergence the evidences of GSKIP as an AKAP which anchor both PKA and GSK3beta, Drp1 was designated as an effector to evaluate the possible roles of GSKIP involved in Drp1-mediated at Ser637 and Ser693 of mitochondrial functions. Our data showed that overexpressed GSKIP wt significantly prevents mitochondrial fragmentation from H2O2 insult when compared with GSKIP V41/L45P, but not L130P. The data also suggested that GSKIP might act as an AKAP to promote PKA to phosphorylate Drp1 at Ser637 rather than GSK3beta at Ser693, leading to a deceased mitochondrial fission and conferring neuroprotection by H2O2 stress. Taken together, we proposed the interplay among GSKIP, PKA, GSK3beta and Drp1 in neuroprotection and leading approaches toward rational therapeutics of neurodegenerative diseases.

SW03.S13–27

The extracellular nuclear acids in blood of patients with different clinical forms of chronic obstructive pulmonary disease (COPD)

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The aim of the work was to study the extracellular nuclear acids (RNA and DNA) concentrations in blood of patients with different clinical forms of COPD. Patients were divided into two groups. Twelve patients with COPD, moderate severity mixed form (emphysematos and bronchial), exacerbation, respiratory insufficiency of grade 2 were included in first group. Twelve patients with COPD, moderate severity bronchial form, exacerbation, respiratory insufficiency of grade 2 were included in 2-hd group. The control group consisted of 32 healthy subjects. In plasma of all subjects the concentrations of extracellular RNA, DNA and acid-soluble precursors of nucleic acids were detected following the protocol of L. Markusheva et al (2000).

All patients and healthy subjects had received the full information on probable inconveniences at the blood sampling before giving their written informed consent.

The results obtained demonstrated the significant increasing of extracellular RNA and acid-soluble precursors of nucleic acids in plasma of COPD patients in compared of healthy ones. Extracellular RNA levels in plasma of COPD patients exceeded the control value by 2.4 and 3.8 times, p < 0.001. In plasma of COPD patients the concentrations of the acid-soluble precursors of nucleic acids were higher than in control ones (by 2.4 and 3.8 times, p < 0.001). Extracellular DNA levels in plasma of COPD patients exceeded the control value by 1.75 (p < 0.001) and by 1.25 times, respectively (p < 0.01).

Taken together, our results obtained demonstrated the augmentation of extracellular nuclear acids releasing in blood of patients in dependence of clinical forms of COPD. In our opinion, the extracellular nuclear acids releasing was determined with activated phagocytic cells and amplification of inflammatory process. We suggested the augmentation of acid-soluble precursors of nucleic acids to be connected with activation of endonucleases in blood of patients.

The possible ways of the participation of extracellular nuclear acids in the mechanisms of progression of COPD were offered.

SW03.S13–28

Short-term biochemical responses of gibel carp white muscle due to silicon quantum dots exposure

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To study the potential toxicity of silicon-based quantum dots, lots of 10 fishes intraperitoneally injected (five with QDs and five with NaCl 0.7%) were sacrificed after 1, 3, and 7 days and the tissues were prelevated.

The effects on white muscle were investigated by analyzing the level of following parameters: reduced glutation (GSH), malondialdehyde (MDA), advanced oxidation protein products (AOPP), protein thiols and protein reactive carbonyl groups. Superoxid dismutase (SOD), catalase (CAT) and metalloproteinases (MMPs) activity were also evaluated.
Due to their intrinsic fluorescence, the bioaccumulation of QDs was highlighted in white muscle tissues by fluorescence microscopy. Biochemical assays revealed that GSH level significantly increased after 1 and 3 days of exposure by respectively, 85.3 and 25.4%. Seven days later, GSH levels were similar to controls. MDA concentration rose after 3 days by 46.9% and remained at the same level after 7 days. Protein thiol levels significantly decreased by 6.7 and 8.1% after 3 and 7 days, whereas advanced oxidation protein products increased by 12.7, respectively, 28.1% in the same time intervals. The protein reactive carbonyl groups were raised only after the first day of exposure and returned to the control level later on. SOD specific activity increased up to 48% after 7 days, while CAT activity increased by 328, 176, and 26% after 1, 3, and 7 days of treatment. The activity of pro-MMP-2 decreased in a time-dependent manner and at MMP-9 level the modifications were less pronounced.

Taking into account the elevated GSH level, the cessation of lipid peroxidation, and the low level of oxidative alterations of proteins in the white muscle, it appears that this Carassius gibelio tissue has the capacity to cope with oxidative stress caused by silicon-based quantum dots, possibly due to the adapted response of the antioxidative enzyme during the exposure.

**SW03.S13–29**

**Toxicological aspects of PANC-1 cells exposure to iron oxide dextran-covered nanoparticles**

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Superparamagnetic nanoparticles (SPION) have currently become very popular as Magnetic Resonance Imaging (MRI) contrast agents because of their strong magnetic efficacy and because they are composed of biodegradable iron. Our purpose was to evaluate the degree of dextran-coated SPION uptake, their MRI efficacy, intracellular localization and potential toxic effects produced on human pancreatic carcinoma cells line (PANC-1) after 24, 48 and 72 h of exposure.

SPION were obtained by coprecipitation technique. PANC-1 cells were exposed at 0 (control), 28 and 56 µg Fe/ml of SPION for 24, 48 and 72 h. The iron content in PANC-1 cells was determined by two methods: one based on NMR relaxometry and the other on Perl's reaction. The SPION cellular uptake and localization were highlighted by DAB-enhanced Prussian blue staining and by lysosomal fluorescent labelling. SPION labeled cells were analysed by MRI at 7T (Bruker Pharmascan). The potential toxic effects were evaluated by ROS production, and the malondialdehyde (MDA) and glutathione (GSH) measurement.

A significant increase of cellular iron content was observed starting with 48 h of exposure reaching up by 2.6 fold for the low concentration and by 5.3 fold for the high concentration compared to control after 72 h of treatment. MRI and microscopy images confirmed the cellular uptake. The lysosomes number increased due to SPION localization at this level in a time-dependent manner and it was almost identical for both SPION concentrations (by 1.6 fold after 48 h and by 2.6 fold after 72 h). Accumulation of SPION produced a strong negative contrast on T2-weighted images starting with 48 h. A time dependent increase in ROS production by 1.6, 1.9 and 2.4 fold was observed for the respective incubation times at low. For a double dose, ROS levels were by 2.2, 2.5 and 3.9 fold higher than control at the same intervals. An increase in the amount of MDA by 1.1, 1.4 and 2.3 fold after 24, 48 and 72 h correlated with the decrease in GSH levels (by 1.35, 1.83 and 1.92 fold respectively) was observed only for the 56 µg Fe/ml dose.

To conclude, our results suggest that dextran-coated SPION were progressively accumulated in PANC-1 cells and allowed the contrast increase of MR images. Starting with a dose of 56 µg Fe/ml, the production of ROS was inefficiently counteracted and lipid peroxidation occurred.

**SW03.S13–30**

**The effect of external thiamine on the antioxidative potential of baker’s yeast**

**Saccharomyces cerevisiae** under abiotic stress conditions

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Thiamine (vitamin B₁) is an essential component of human diet and its decreased level can lead to severe health disorders, affecting primarily the cardiovascular and nervous systems. This is mainly owing to the crucial role of thiamine diphosphate (TDP) that serves as a cofactor for key enzymes in the carbohydrate metabolism. Recent findings suggest another important function of this vitamin in the responses of organisms to stress conditions; however, the mechanisms of this novel role of thiamine has not yet been characterized.

Therefore, in the present study, we aimed primarily at explaining the impact of thiamine on the cellular defence system in the model eukaryote, baker's yeast *Saccharomyces cerevisiae*, with a special focus on the localisation of selected antioxidant enzymes within the cell. The yeast cultures were subjected to the most common types of abiotic stress (oxidative, osmotic and thermal) and the changes in gene expression of antioxidant enzymes were determined by Real Time PCR. Under all selected conditions, the absence of thiamine in the growth medium has led to an increased expression of cytosolic genes *CTT1* and *GPD1* (by more than 350%) as compared to the growth in the standard thiamine-rich medium. The effect was also clearly observed for mitochondrial and peroxiosomal proteins *SOD2* and *CTA1*, especially under oxidative stress (700% and 200% increase, respectively). Intriguingly, this effect seemed to be reversed at the periphery of the cell, with a decreased expression for both plasma membrane and cell wall stress-protecting proteins (e.g. *HSP12, HSP150*). In order to determine the direct antioxidative properties of thiamine, the results were corroborated with the quantification of free radicals and protein oxidation levels.

Taken together, our results strongly support the involvement of thiamine in alleviating stress responses, indicating, however, that the mechanism of its action seems to be multifactorial and requires further investigation.

**SW03.S13–31**

**The structure and fine tuning of hsp70 promoters in different animal species with contrasting thermal habitats**

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Heat shock proteins (Hsp) provide cellular and whole body adaptation of animals to various adverse environmental conditions.
Hsp70 is apparently the major player underlying biological adaptation in all organisms studied so far.

In all animals studied, heat shock genes regulatory regions include several conservative promoter elements HSEs (heat shock elements) that are necessary for binding of heat shock transcription factor (HSF) [1]. The promoters regions of hsp genes are extremely conserved and, hence, it was generally accepted that they are universal and can operate in species belonging to different phyla.

In the present work we sequenced hsp70 genes from a highly thermotolerant Diptera species (Stratiomyss singularis), the larvae of which inhabit Crimean lakes [2]. The comparative analysis revealed characteristic differences in the promoters of this thermotolerant species and those of D. melanogaster. We also performed the investigation of promoters ‘strength’ in Diptera cell culture exploring in vitro luciferase reporter assay. The analysis demonstrated significantly higher strength of D. melanogaster promoters in spite of the fact that HSEs are present in both species. Probably these drastic differences in the promoters strength are due to specific binding of GAF which is also necessary for efficient hsp genes induction and functioning.

Furthermore, we investigated the structure and strength of hsp70 promoters in two mammalian species (humans and camels). It was shown that their promoters also exhibit specific differences and camel’s promoters provide higher levels of hsp70 transcription under normal physiological conditions. However, the differences between human and camel’s promoter strength are not so significant after heat shock treatment. Therefore, in our study we demonstrated species-specific promoter structure and fine tuning of hsp genes which contradicts previously accepted dogma postulating universal robust non-specific pattern of hsp genes regulation.

References


SW03.S13–32
The heat shock transcriptome of the legume microsymbiont Mesorhizobium sp.
MAFF303099

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Diazotrophic bacteria are able to uptake atmospheric nitrogen and metabolize it, a process known as biological nitrogen fixation (BNF). Some of these bacteria, named rhizobia, establish symbioses with legume plants, providing them with N-compounds. These symbioses are particularly important for agricultural species, since N is a macronutrient often limiting crop production. Compared to N fertilizers, BNF is an environmental friendly process to supply N to agro-systems. As soil bacteria, rhizobia are exposed to biotic and abiotic factors. The present study applied a genome-wide approach to investigate the response to heat stress of a Mesorhizobium strain able to establish symbiosis with Lotus sp.

The analysis of the Mesorhizobium sp. MAFF303099 transcriptome allowed the identification of 2186 genes (about 30% of the protein-coding genes) that were differentially expressed after a heat shock. The number of genes found to be underexpressed (1584 genes) was much higher than the number of overexpressed genes (602 genes). In both cases, genes from the three replicons – chromosome, plasmid pMLA and plasmid pMLB – were involved. Rhizobial genomes typically harbour several copies of the operon encoding the GroESL chaperone system, usually with different regulation mechanisms (Alexandre & Oliveira, in press). MAFF303099 genome encodes five groESL operons, from which only one seems to play an important role in the heat shock response, as its expression increased approximately 55-fold under this condition. One operon encoding two small heat shock proteins showed the highest overexpression detected in this study, which is consistent with the heat shock response of another rhizobium previously studied. The expression of some genes involved in host nodulation and nitrogen fixation was also changed following heat shock. Most of these genes were underexpressed, in particular the fix genes belonging to a second operon outside the symbiosis island.

The present study contributes to the current knowledge on rhizobia response to stress, nevertheless further studies are in course to understand the role of individual genes and the mechanisms regulating these responses.

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Reference


SW03.S13–33
Bioaccumulation of Si/SiO2 QDs and the oxidative stress biomarkers assessment in the kidney tissue of Carassius auratus gibelio

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The impact of medium-term exposure to silicon quantum dots on Carassius auratus gibelio was evaluated through changes of selected parameters considered as oxidative stress biomarkers. The distribution of QDs and the histological effects on renal tissue were highlighted by optical microscopy. In this study we used Si/SiO2 QDs with a log-normal size distribution between 2.75 and 11.25 nm. Biochemical parameters like malondialdehyde (MDA), reduced glutathione (GSH), oxidative protein modifications (advanced oxidation protein products, carbonyl and sulfhydryl protein groups), protein expression of Hsp 70 and MMP 9 activity were evaluated in kidney of Carassius auratus gibelio intraperitoneally injected with 2 mg QDs/kg body weight. After 1, 3, and 7 days, 10 fish (five IP injected with QDs and five injected with NaCl 0.7%) for each time point were sacrificed under anesthesia and tissue were prelevated.

The reduced glutathione GSH concentration decreased by 66%, 60% and 47% respectively after 1, 3 and 7 days of treatment compared to controls. The MDA level significantly increased by 97% after 1 day of exposure, reaching the maximum level of 288% higher than the control after the third day.
Protein thiol levels significantly decreased by 28%, 33% and 43% respectively after 1, 3 and 7 days of treatment whereas advanced oxidation protein (AOPP) increased significantly in the same intervals compared to control.

The protein reactive carbonyl group level was significantly up-regulated after 3 and 7 days of exposure by 122% and 115% respectively compared to control levels. The Hsp 70 expression in the renal tissue increased in a time-dependent manner by 1.33, 1.65 and 1.88 fold, after 1, 3 and 7 days, respectively, compared to controls. After treatment with QDs in renal tissue no significant variations were observed in MMP9 activity. Taking into account all our data, it appears that a dose of 2 mg silicon-based QDs per kg body weight IP injected in C. auratus gibelio caused moderate damage to renal tissue after 7 days of exposure.

**SW03.S13–34**

Regucalcin, an apoptosis regulator in spermatogenesis

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Regucalcin (RGN) is a calcium (Ca²⁺)-binding protein playing an important role in intracellular Ca²⁺ homeostasis. It has also been shown that RGN overexpression suppresses cell death and apoptosis induced by noxious stimuli, and accordingly, RGN knockout mice are more susceptible to undergo Fas-mediated apoptosis. Spermatogenesis is a complex process depending on the crucial action of androgens, which are known as survival factors inhibiting apoptosis of male germ cells. Recently, we have demonstrated that androgens up-regulate RGN expression in the testis, linking this protein with the control of testicular apoptosis. The objective of the present study is to analyze the role of RGN regulating cell death in rat testis. Ex vivo cultures of seminiferous tubules (SeT) from transgenic rats overexpressing RGN (Tg-RGN) and wild-type (Wt) controls were maintained for 48 h in the presence or absence of apoptosis-inducer thapsigargin (Thap, 10⁻⁷ and 10⁻⁶ M). Expression analysis of key regulators of apoptosis was performed by real-time PCR and Western Blot. An end-point of apoptosis was also studied by measurement of caspase 3 enzymatic activity. The observed diminished activity of caspase 3, which was accompanied by decreased transcription of caspase 3 gene, and increased mRNA and protein expression of anti-apoptotic Bcl-2 protein, suggests a suppression of Thap-induced apoptosis in SeT of Tg-RGN rats. Although caspase 9 mRNA was increased in Tg-RGN, no differences were observed at protein level. Also, no differences on protein levels of AIF were found between Tg-RGN and Wt groups. It is also noteworthy the fact that mRNA expression of proapoptotic proteins p53 and p21 is strongly decreased in Tg-RGN treated with Thap. Therefore, the present results indicate RGN as an important target in the control of testicular apoptosis, acting as a germ cell survival factor, alone or as a mediator in androgen signaling pathways.

**SW03.S13–35**

Regulation of pirin expression as a mechanism enabling cross-talk between NRF2 and other transcription factors

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Pirin (Pir) is a nuclear protein isolated and characterized as an interactor of the NFIX transcription factor. Subsequently, it was revealed that Pir also forms complexes with Bcl3 and NF-κB1 (p50) and therefore, may be implicated in the regulation of the NF-κB-related transcription. Some studies have linked Pir orthologues with apoptosis and stress responses, whereas other have pointed out the deregulation of Pir expression in several human tumors.

Using an in vivo model of oxidative stress, namely, Sod1-deficient mice we have found that Sod1⁻/- mice have a significantly elevated level of Pir mRNA in the spleen and kidney comparing to their wild-type counterparts. To investigate factors responsible for regulation of Pir expression we have cloned DNA fragment containing putative promoter of human Pir gene. Several plasmids were constructed with luciferase transcription under the control of different Pir promoter fragments. Analysis of luciferase expression from the resulting constructs in HeLa cells showed that the most important part of Pir promoter is highly conserved sequence located 280 bp downstream transcription start site including antioxidant response element (ARE). Since ARE is a well-known binding site for NRF2 transcription factor, we analyzed Pir expression as well as luciferase expression from aforementioned constructs in HeLa cells transfected with anti-NRF2 siRNA. Both types of experiments confirmed that Pir expression is dependent on NRF2 transcription factor. This was further confirmed by ChIP experiment showing that NRF2 binds to ARE located 280 bp downstream transcription start site of Pir gene.

The results of our experiments suggests that via regulation of pirin expression NRF2 can influence NF-κB (and possibly other transcription factors) activity which may contribute to a more precise regulation of the cellular response to stress.

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**SW03.S13–36**

Zinc homeostasis and eryptosis

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Background: Zinc (Zn²⁺) is an essential trace element that is required for a wide range of biological processes, including cell proliferation and differentiation. The physiological concentration of Zn²⁺ in serum is 2–15 μM, while free Zn²⁺ in most cells is extremely low (<1 nM). However, intracellular zinc homeostasis is sensitive to pathophysiologival environmental changes. The aim of this work is clearing of interaction of zinc homeostasis with induction of erythrocyte programmed cell death (eryptosis).

Observations: Using specific ionophore, intra- and extracellular zinc chelators was determined the existence of the specific receptors on the cell surface and cellular stores responsible for the maintenance of zinc homeostasis in erythrocytes. At the same time was shown that increase of intracellular labile Zn²⁺ level over 70 nM results in induction of eryptosis and Zn²⁺ cytotoxic effects (cytosolic esterase activity inhibition, phosphatidylserine redistribution on the cell membrane surface) is caused by intracellular molecular mechanisms lead to Zn²⁺ release from cellular stores. Also was established that the disturbance in ‘prooxidant/antioxidant’ balance is possible trigger of eryptosis initiation.

Conclusion: The received results testify about existence of regulation mechanisms of zinc homeostasis in human erythrocytes and the fine concentration ‘border’ between essential and toxic properties of Zn²⁺, failure of which can lead to the triggering of the processes of programmed cell death.
Selection of the appropriate housekeeping genes, known to have stable and unaltered expression levels in response to a wide range of stressors, chemicals, diseases, climatic changes, xenobiotics and environmental effectors, is the most important pre-phase experiments to perform in a wider context relative quantitation study which requires comparative results. A candidate housekeeping gene, i.e. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is expected to be expressed constantly in the cell, and PCR method development and selection is evaluated, based on this parameter, in the different organs and tissues to be investigated. The gills are the first organs to react in response to xenobiotics in fish and the liver is the major organ with detoxification enzyme systems activated/ altered when exposed to stressors and toxicants. Although glyceraldehyde-3-phosphate dehydrogenase gene is a promising candidate housekeeping gene, recent research has shown that its constant expression levels are not universal and therefore cannot be applicable/useful in all species and also in most organs. We studied GAPDH, EF1α (Elongation factor 1-α) and β-actin as candidates and very promising genes in the common carp (Cyprinus carpio) gill and liver tissues and employed them to develop a method for the assessment of Hsp70 expression levels in response to dibutyl phthalate (DBP; a highly abundant, aquatic toxicant chemical plasticizer and organic pollutant with endocrine disrupting activity) exposure. Carp were obtained from State Hydraulic Works General Directorate (DSI, Turkey) and exposed to a sub-lethal concentration of DBP (1 mg/l) for 4, 24 and 96 h. A control group was included. RNA quantification of gills and liver tissue for inducible Hsp70 levels were carried out using a two-step real-time RT-PCR with special primer pairs designed for carp Hsp70 mRNA and housekeeping candidates. We evaluated GAPDH, EF1α and β-actin Ct values in each sample and used ‘Bestkeeper’ special programme to calculate CV (coefficient of variation) and ‘power’ values. Results showed that β-actin was the best housekeeping gene in gills and liver of the common carp and the least reliable one was GAPDH. These results provide data for gene expression analysis studies in the carp as a model and standard test organism, and support the literature with results of Hsp70 expression analysis in response to DBP exposure, showing DBP to be an aquatic and environmental pollutant with toxic response at the cellular and molecular levels. In conclusion, our data report that Hsp70 expression is dramatically down-regulated with 2–4 folds in 4, 24 and 96 h exposure groups. In addition, the model animal approach in the present study unequivocally shows the need for a case-by-case housekeeping gene selection for each organism studied.
sive traits or high stress response in general, an element that accompanies the development of depressive features in humans and animals, remains unknown.

In the present study, we have employed two widely used models of depression, the forced swim paradigm, and a model of stress-induced anhedonia, in C57BL6N mice. In each model, sub-groups with pronounced manifestations of depression-like behavior were defined, in accordance to selected criteria of prolonged floating behavior (>185s), or lowered sucrose preference (<65%), respectively. Groups of mice that have received chronic antidepressant treatment, were included in the analysis as well. Enzyme-linked immunosorbent assay (ELISA) was used to estimate the levels of GSK3β in freshly frozen hippocampus tissue. We found no significant changes in phosphorylated 9-serin GSK3β (pS9GSK3β) between groups with high and low duration of floating, while pS9GSK3β was affected by imipramine in this test. Chronically stressed anhedonic mice with a high duration of floating behavior has a tendency to a significant difference in the pS9GSK3β, as compared to stressed non-anhedonic mice, whose scores of floating behavior were not distinct from those of non-stressed controls. These data were in line with previously obtained findings of increased hippocampal expression of GSK3β in stressed mice with anhedonia, as demonstrated by Illumina gene expression microarray method.

Together, our data suggest the specificity, with regard to general effects of chronic stress, with the changes in brain levels of GSK3β, in animals susceptible to depressive-like changes, in the chronic stress depression model. Up to now, this was not observed in the forced swim test. More studies are required to demonstrate whether the levels of expression or regulation of GSK3β activity by phosphorylation are functionally important during stress and depressive-like states.

**SW03.S13–40**  
**Vascular endothelial growth factor (VEGF) resistance in human monocytes: implications for impaired arteriogenesis in diabetes mellitus**  
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Arteriogenesis/collateral growth is a process encompassing the growth of pre-existing collateral blood vessels to form functional arteries, which occurs as a compensatory mechanism when there is severe limitation of blood supply due to plaque formation in the arteries. Among the arteriogenic cells, monocytes play a vital role. VEGF and its receptors are closely involved in the process of arteriogenesis by modulating the responses of monocytes. Proper arteriogenesis development is linked to 36% reduced mortality risk, it is important to find new means to induce arteriogenesis.

Cardiovascular risk factors such as diabetes mellitus negatively influence arteriogenesis. This defective arteriogenesis is speculated to be due to the dysfunction of monocytes, leading to the abnormality of these cells to home to the sites of vessel growth. We could show that monocytes from diabetic individuals are defective in their migratory potential towards VEGFA and PLGF-1, a condition termed as ’VEGF resistance’. Hyperglycemia caused ligand-independent, constitutive activation of ERK, p38 and PI3K/AKT. The abnormal functionality of monocytes in hyperglycemic conditions is linked to this hyperactivated state. Molecular mechanisms leading to monocyte dysfunction in diabetes are incompletely understood.

We show that oxidative stress induced by hyperglycaemia is able to up-regulate transient receptor potential canonical (TRPC) channels, thereby enhancing calcium-dependent activation of small GTPase-Rhoa and its downstream effector ROCK. ROCK in-turn is able to phosphorylate and activate AKT in hyperglycaemic monocytes. We also demonstrate a ROCK-dependent stabilization of PTEN in hyperglycaemia. The preactivation of AKT seen in hyperglycaemic monocytes was found to be due to the aberrant Rho-ROCK activation. By pharmacological inhibition of ROCK, we could significantly block the aberrant activation of AKT and revert monocyte dysfunction. This finding could potentially allow us to appreciate the positive effects of statins, a ROCK inhibitor, with respect to arteriogenesis. Analysing arteriogenesis in vivo by using murine hindlimb ischemia model by employing ROCK1/-/- mice will provide further information of the role of ROCK1. This will allow us to determine whether targeting the molecules responsible for the deregulated signal transduction in diabetic monocytes will reverse monocyte dysfunction and thereby possibly contribute to the therapeutic stimulation of arteriogenesis in diabetes patients.

**SW03.S13–41**  
**Mitochondrial electron transport chain complex III inhibition causes nucleolar disruption via de novo pyrimidine biosynthesis pathway**  
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The main function of the nucleolus is rRNA synthesis and ribosome assembly, but it also acts as a sensor of wide variety of cellular stresses. Impairment of nucleolar function and disruption of its structure occurs in presence of agents that induce tumour suppressor p53, cell cycle arrest, and apoptosis. Mitochondrial electron transport chain (ETC) complex III inhibitor myxothiazol has recently been shown to stabilize p53 and induce p53-dependent apoptosis due to pyrimidine depletion, which occurs via inhibition of dihydroorotate dehydrogenase, an enzyme in the inner mitochondrial membrane that functionally couples ETC with pyrimidine biosynthesis. Pyrimidine depletion may cause rDNA transcription inhibition followed by disruption of the nucleolar structure. To test this possibility, we visualized nucleoli using nucleolar proteins fibrillarin and nucleophosmin fused to EGFP and tagRFP, respectively. We found that ETC complex III inhibitor myxothiazol induces nucleolar disruption. After 6 h incubation nucleolus becomes smaller, fibrillarin partially translates into ring-shaped structures – so called ’caps’ specific for rDNA transcription inhibition – and exits into the nucleoplasm with formation of granules. Supplementation with exogenous uridine restores the levels of intracellular pyrimidines that were depleted by myxothiazol and prevents myxothiazol-induced p53 stabilization. We found that uridine also prevented caused by myxothiazol nucleolar disruption. Addition of exogenous cytidine restores only CTP pools, however, it prevents formation of fibrillarin granules in the nucleoplasm and delays but does not prevent rDNA transcription inhibition as smaller ’caps’ are formed. CTP pool size limits the biosynthesis of phospholipids that are involved in several signal transduction pathways. Our data demonstrate an essential role for CTP synthesis in maintenance of nucleolar structure during ETC complex III inhibition.

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Bilirubin mediated oxidative stress involves activation of antioxidant response through Nrf2 pathway in SH-SY5Y cells

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Bilirubin is responsible for neonatal jaundice and high level of free bilirubin (Bf) can lead to kernicterus. The molecular mechanisms by which Bf induces selective cell injury are incompletely elucidated and oxidative stress (OS) is suggested as a hallmark of Bf induces neurotoxicity. The Nrf2 is a powerful sensor for cellular redox state and activated directly by OS or indirectly by stress protein kinases. It translocates to nucleus, binds to Antioxidant Response Element (ARE), and enhances the up-regulation of antioxidant genes such as: HO-1, FTH, NQO-1, γGCL-m, and xCT.

SH-SY5Y neuroblastoma cells were incubated with Bf (140 nM). Intracellular ROS were monitored by H2DCFDA. Nuclear Nrf2 proteins were detected by Western blot and mRNA expression was analyzed by qRT-PCR. Cells were transfected with reporter gene (ARE-GFP) and GFP signal was detected using Fluorescence microscopy. Cells transfected with Nrf2 siRNA or control siRNA were treated with Bf and genes analyzed by qRT-PCR. Cells were pre-treated with antioxidant (0.5 mM NAC) or specific signaling pathways inhibitors 1 h before Bf exposure.

SH-SY5Y cells showed increased levels of intracellular ROS after 15 min of Bf exposure. Bf mediates nuclear accumulation of Nrf2 after 3 h and increases GFP signal (after 32 h) in cells transfected with ARE-GFP reporter while no signals were detected in controls. Bf induces mRNA expression of xCT (9 fold), γGCL-m (3 fold), HO-1 (30 fold), NQO-1 (5.3 fold), and FTH (3 fold) at 24 h. Nrf2 siRNA decreases mRNA expression of HO1 (75%), NQO1 (56%), and FTH (40%) compared to controls upon Bf treatment, while no changes were detected on the expression of γGCL-m, and xCT. Nrf2 related HO-1 induction was reduced to about 70% in cells pre-treated with NAC and in cell pre-treated with PKC or MEK1/2 inhibitors compared to controls.

For the first time we demonstrate that Bf mediates nuclear accumulation of Nrf2 and induces transcriptional activation of ARE. HO-1, NQO1 and FTH are up-regulated by Bf at Nrf2 dependent manner. HO-1 induction by Bf involves oxidative stress and stress response protein kinases (PKC and MEK1/2).

The heat shock protein TRAP1 is involved in translational control: a novel role in the quality control of mitochondrial proteins

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TRAP1 is the main mitochondrial member of the HSP90 family, induced in most tumor types and involved in stress-adaptive responses of cancer cells. The organelle-directed regulation by this chaperone of folding and stability of selective proteins involved in mitochondrial homeostasis, such as Cyclophilin D and Sorcin, is pivotal for the control of tumor cell’s protoxicosis, leading to resistance to apoptosis. Notably, we have previously demonstrated that TRAP1 is also localized in the endoplasmic reticulum, where is involved in an extra-mitochondrial quality control of some nuclear-encoded mitochondrial client proteins. This study demonstrates for the first time that TRAP1 is associated to ribosomes and several translation factors in colon carcinoma cells and, remarkably, is found co-upregulated with some components of the translational apparatus (eIF4A, eIF4E and eEF1G) in human colorectal cancers. We show that TRAP1 regulates the rate of protein synthesis through the GCN2-eIF2α pathway, favoring the activation of the kinase GCP2, with consequent phosphorylation of eIF2α and attenuation of cap-dependent translation. This enhances the synthesis of selective stress-responsive proteins, as the transcription factor ATF4 and its downstream effectors BiP/Gp78, the cystine antipporter system xCT, and the autophagy marker LC3, thereby providing cell’s protection toward ER stress, oxidative damage and nutrient deprivation. Accordingly, TRAP1 silencing sensitizes cells to apoptosis induced by novel antitumor drugs that inhibit cap-dependent translation, such as ribavirin or 4EGI-1, and reduces the ability of cells to migrate through the pores of transwell filters. These new findings candidate TRAP1 network as a target to develop novel anti-cancer strategies.

Neurons have an active glycogen metabolism that plays a key role in the tolerance to hypoxia

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Glycogen in the brain has been almost exclusively located in astrocytes. However, neurons express Glycogen Synthase (GS), the only enzyme capable of its synthesis in mammals [1]. Moreover, in several pathologies, polymers of glucose, normally referred to as polyglucosan bodies, accumulate in neuronal tissue [2], and some studies have shown that an excess of glycogen accumulation in neurons triggers apoptosis [3]. These data raise the question why neurons have the enzymatic machinery to synthesize glycogen if they do not accumulate the polysaccharide.

We have worked with neuronal cultures from mice and genetically engineered Drosophila melanogaster and mice that accumulate glycogen or lack GS specifically in neurons.

We show that there is an active glycogen synthesis under normal conditions. In addition, neurons express the necessary machinery to degrade the polysaccharide and degrade it under hypoxia conditions. The glycogen degradation machinery is mediated through the expression of glycogen phosphorylase (GP). Neurons express the brain isoform of the enzyme, but not the muscle, as astrocytes do. This isoform is present in neuronal cultures, as well as in neurons from adult mice brain slices.

Finally, we have demonstrated that glycogen metabolism is part of the protection machinery the neuron activates for tolerating the hypoxia conditions. Consequently, neurons without GS have a higher mortality rate than those who actively express the enzyme. In Drosophila melanogaster, flies with reduced GS levels specifically in neurons have an impaired phenotype in their response to hypoxic conditions.

In this study we illustrate the presence of glycogen in neurons and demonstrate that neuronal glycogen metabolism plays a crucial role for neuronal survival. We show that neurons actively synthesize glycogen and prove that neurons have a strong capacity of glycogen degradation both in neuronal cultures as well as in mice and flies.

References
SW03.S13–45
Attenuation of hepatic damage by PARP-1 inhibitors treatment under diabetes in rats
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The present study was designed to investigate the impact of experimental diabetes on the liver dysfunctions. We also tested whether 1,5-isoquinolinediol (ISO), a recent generation PARP inhibitors and nicotinamide (NAm), as drug with the wide range of metabolic effects can influence liver impairments associated with diabetes in therapeutic doses. All studies were carried out after 10 weeks of diabetes (streptozotocin, 55 mg/kg of body weight, i. p.) in male Wistar rats treated for 14 days with or without ISO (3 mg/kg, i. p.) and NAm (100 mg/kg, i. p.). The experimental diabetes caused a hyperglycemia and increase ROS production in rat blood leukocytes. Nicotinamide decreased of level ROS production. Notably, that ISO supplementation to diabetic rats had no effect on blood glucose level while NAm caused a slight lowering effect. In diabetic rats we established reduction of the liver NAD+/NADH and NADP+/NADPH ratios in diabetic liver were lowered 2.77- and 2.81-fold respectively vs. control. It was shown more than 2.5-fold increases in mean free cytosol NADP+ and NADH. It was found a strong normalizing effect of NAm on redox state of diabetic liver were lowered 2.77- and 2.81-fold respectively vs. control.

SW03.S13–46
The role of membranes in the heat-stress management
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Important diseases (cancer, type 2 diabetes, etc.) are known to be associated with abnormal level of heat shock protein (HSP) molecular chaperones and characteristic membrane defects. This presentation aims to establish a mechanism for the possible interconnection between specific changes of lipid composition, fluidity and microdomain organization of plasma membranes and the simultaneously altered (dysregulated) expression of HSPs. Exposure of cells to non-proteotoxic membrane fluidizers, cholesterol depleting agents, or non-proteotoxic drug candidates which interact specifically with certain membrane domains can strongly modulate the expression of heat shock proteins. Monitoring the surface membrane microdomains by confocal- and ultrasensitive single molecular microscopy we established a relationship between specific distribution of lipid nanostructures (‘rafts’) and the concomitant changes in the level, profile and cellular distribution of HSPs. A comparative lipidomics study explored key lipids with the potential to activate of HSP signaling pathways. Drug candidates, capable to refine HSP profile by targeting specific membrane microdomains, – with considerable therapeutic benefit-, will also be discussed. A subpopulation of HSPs is membrane associated: via their specific lipid interactions the moonlighting HSPs can control major attributes of the membranes, like fluidity, phase state or curvature. The membrane microdomain associated HSPs can also participate in the orchestration of distinct raft-associated signaling platforms: thus, membrane association of HSPs can refine hsp gene expression.

References

SW03.S13–47
The impact of peritoneal dialysis fluid exposure on O-GlcNAc modification and resulting changes in stress response and survival of mesothelial cells
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Peritoneal dialysis (PD) is an alternative to haemodialysis and the only modality to treat end-stage renal failure in newborns and infants. PD uses the mesothelial cells (MC) of the peritoneum for removal of uremic toxins and excessive water. Because of their physicochemical properties peritoneal dialysis fluids (PDF) harm the MC layer and lead to loss of dialysis function. The two major reasons for bioincompatibility of PDF are high glucose and glucose degradation products, which are both known to influence glycosylation. Because modification of proteins with O-GlcNAC is relevant for regulatory functions such as the stress response the aim of this study was to examine the effect of PDF on O-GlcNAcylation, its relevance for cell survival and glutamine-mediated cytoprotection of MC.

Primary and immortalised MC were treated with PDF and the influence on the abundance of O-GlcNAc was analysed by Western blot and immunofluorescence. The impact of the modulation of O-GlcNAcylation with specific inhibitors on the outcome after PDF exposure was additionally analysed by viability assays. Furthermore a 2D-gel based screen was employed to analyse differential abundance of MC proteins modified by O-GlcNAc.

Exposure of MC to PDF leads to significantly increased abundance O-GlcNAc modified proteins. Inhibition of O-GlcNAc-Modification is associated with decreased viability and expression of P70. Increased levels of O-GlcNAc in turn lead to improved viability of cells following PDF exposure. Addition of glutamine to PDF leads to higher abundance of O-GlcNAc, enhanced survival and increased P70 expression. The total proteome of MC shows limited differences in protein abundance, whereas O-GlcNAc specific 2D immunoblotting reveals distinct differences in levels of O-GlcNAc-modified proteins after PDF treatment.
The fibrinogen was precipitated by sodium sulfate.

Experiments were carried out on white outbreed male rats. The level ofacting noise was 91 dBA (duration of noise action 7, 30, 40 days; 8 h per day). Blood was taken by cardiopuncture. The fibrinogen was precipitated by sodium sulfate. POM evaluation was carried out based on dinitrophenylhydrazine (the interaction products of protein oxidized aminoacid residues with 2,4-dinitrophenylhydrazine) content, registered on spectrophotometer SP-26 with wave lengths 356, 370 and 430 nm and was expressed in nmol of dinitrophenylhydrazine/mg protein. Clotting velocity was determined by plasma recalcification time. Statistical analysis was done by Graph Pad In Stat Software.

The data obtained reveal significant increase of white rats plasma proteins and fibrinogen oxidation under the noise action, intensity of which depends both on the duration of noise action and studied parameter. The most interesting is drastic increase of fibrinogen oxidation after 30 days of noise action which accompanied with the increase of LDL-Ch and TAG, decrease of HDL-Ch, lipid peroxidation intensification in plasma and clotting time increase. The high level of correlation was found out between received data. Summing up the results, we conclude that noise action leads to oxidation of plasma proteins, fibrinogen, shifting in the LP-Ch content, which play an important role in the development of atherosclerosis and cardiovascular diseases.

SW03.S13–50
Lipid-protein intermolecular interactions in erythrocyte membranes of white rats after high level noise action
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Our previous studies revealed oxidative stress development, particularly significant changes in the intensity of lipid peroxidation, alfa-tocopherol content and lipid composition in the brain mitochondrial and erythrocyte membranes (EM), depending on both the sex of animals and the duration of noise action. The goal of this investigation is to reveal the effects of white noise action (91 dBA, 20–20 000 Hz) on the lipid-protein intermolecular interactions in EM of white rats using fluorescent probes 1-anilinonaphthalin-8-sulphonate (ANS) and pyrene. ANS is believed to strongly bind cationic groups of proteins through ion pair formation and extensively utilized fluorescent probe for the characterization of protein binding sites. Pyrene being apolar in nature, is located in the hydrophobic lipid region of the membrane. The ratio of the monomer to excimer intensity of the embedded pyrene is an index of fluidity parameter of its environment and also of its magnitude of incorporation. Experiments were carried out on white outbreed male rats. Blood was taken by cardiopuncture. During the in vivo experiments the rats preliminary were exposed to 1 h noise action. For in vitro experiments the erythrocyte ghosts obtained from the blood of intact animals were divided into different groups of samples, one of which served as control. Other samples underwent to noise action before and after incubation with ANS and pyrene. It was shown that high level noise influence leads to the decrease of the rate constant of ANS binding with ghosts, accompanied with the decrease in the binding centers number, which is evidence of the changes in the membranes surface charge. The results of fluidity and polarity study of EM revealed dependence of the intensity and direction of changes in the inner part of membrane, which were less expressed compare with the surface changes. Obtained results indicate that the influence of high level noise causes the changes in the lipid-protein interaction which can be the result of molecular reconstruction in the EM as a result both of oxidative stress development and direct action of acoustic waves.
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mapped within its central domain (IDD). This domain is also characterized as disordered one. Interestingly this site within the coilin IDD also contains a cluster of positively charged amino acids. According to the current concept, disordered regions of proteins possess a high potential for interactions with various macromolecular partners. A possible model for interactions between the viral proteins and cellular nuclear protein is discussed. This work was supported by the RFBR grant 13-04-01467.

Reference

SW03.S13–53

The effects of melatonin on kidney nitric oxide-ADMA pathway in fructose-fed rats

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Fructose is found naturally in many sources, such as fruits and honey, in relatively small quantities, however it has become an important part of our diet due to its increasing usage in food industry. Emerging evidences suggest a strong link between high fructose consumption and increased prevalence of metabolic syndrome (MetS). Melatonin, which is primarily synthesized by the pineal gland as well as many other tissues, is also known to be a strong antioxidant and cell protectant in addition to its chronobiologic roles. In our study, we aimed to demonstrate a metabolic syndrome model in rats by high-fructose consumption, investigate the alterations of the renal tissue Nitric Oxide-ADMA pathway and search for the effects of melatonin. Thirty-two male adult Sprague-Dawley rats were randomly divided into four groups (n = 8): (i) Control group, (ii) Fructose group, (iii) Melatonin group, (iv) Fructose + Melatonin group. Fructose was given as 20% solution in tap water to drink ad libitum and 20 mg/kg/day melatonin was administered by oral gavage. Systolic blood pressures were measured at the beginning, at the end of the 4th and 8th weeks. After the experimental period of 8 weeks, rats were sacrificed under anesthesia and to test for the metabolic syndrome criteria, serum glucose, lipid profile and insulin were determined. Renal nitrate-nitrite (NOx), ADMA, Arginine concentrations and endothelial (eNOS) and inducible (iNOS) nitric oxide synthase protein levels were investigated. Our results showed that fructose consumption significantly increased systolic blood pressure, serum triglyceride and insulin levels and caused insulin resistance (metabolic syndrome criteria). Renal NOx levels were found to be non-significantly decreased only in fructose group compared to control group. NOx concentrations of fructose + melatonin group were significantly higher than fructose group. While ADMA and Arginine contents of the kidney tissues of fructose group did not change compared to control group, they were significantly higher in fructose + melatonin group compared to both control and fructose groups. eNOS protein levels were the same among all groups and iNOS protein was not detected in any of the groups.
SW03.S13–54
Mitochondria-targeted antioxidants prevent TNFα-induced endothelial cell apoptosis
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In endothelial cells TNFα activates secretion of blood coagulation factors, NO-synthase, expression of tissue factors, adhesion molecules, inflammatory cytokines and chemokines, transendothelial vesicular transport, cytoskeleton reorganization, disassembly of cell–cell contacts, and cell death [1]. The increased serum level of tumor necrosis factor-α (TNFα) causes endothelial dysfunction and leads to serious vascular pathologies. The TNFα signaling is known to involve reactive oxygen species. Using mitochondria-targeted antioxidant SkQR1, we have studied the role of mitochondrial reactive oxygen species in TNFα-induced apoptosis of human endothelial cell line EAhy926.

It is found that 0.2 nM SkQR1 prevents TNFα-induced apoptosis, but has no influence on TNFα-dependent proteolytic activation of caspase-8 and Bid, inhibits cytochrome c release from mitochondria and cleavage of caspase-3 and PARP. The SkQ-derived compounds lacking the antioxidant moieties do not prevent TNFα-induced apoptosis. The antiapoptotic action of SkQR1 may also be related to other observations made in these experiments, namely SkQR1-induced increase in Bcl-2 and corresponding decrease in Bax as well as p53. The results indicate mitochondrial reactive oxygen species production is involved in TNFα-initiated endothelial cell death, and implies potential of the mitochondria-targeted antioxidants as vasoprotectors. Probably the vasoprotective action of SkQ1 and SkQR1 explains the therapeutic effect of these compounds observed in renal and brain failure animal models [2,3].

References

SW03.S13–55
Whether DNA alone may activate neutrophils and endothelial cells?
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Neutrophils and endothelium cells are essential components of innate immunity and readily activated by both pathogenic stimuli and damage-associated molecular patterns (DAMPs) derived from damaged cells. Mitochondrial debris components (MTD) including mitochondrial DNA (mtDNA) were recently shown to belong to DAMPs [1].

At the present time there are contradictory reports concerning details of DNA-induced activation of neutrophils and endothelial cells. Some researchers report that pure DNA alone may directly activate these cells, while others use Lipofectamine for endothelial cells in order to deliver DNA to endosomal compartments containing TLR9 [2,3].

In our hands highly purified mtDNA from human endothelial cell line EA.hy926 was unable to increase p38 MAPK phosphorylation or MMP9 activity of human neutrophils that contradicted results of C. Hauser [1]. We argue that standard mtDNA purification procedure using a commercially available kit may result in mtDNA impurity causing activation of neutrophils. Similar results were obtained with endothelial cells where ICAM1 mRNA expression level was nearly the same after mtDNA treatment. However, the cells were readily activated by mtDNA complexed with Lipofectamine. At the same time we confirmed that MTD activate human neutrophils.

We also applied mitochondrial-targeted antioxidant (SkQ1) to prevent neutrophils activation by MTD. We showed that SkQ1 treatment decreases the level of MAPK p38 phosphorylation in response to mitochondrial components. However, MMP9 activity and neutrophils chemotaxis were not significantly affected.

References
Quantum dots (QDs) are nanoparticles with intense fluorescence emission and resistance to photo bleaching, however their great potential in biomedical and research imaging is limited due to toxicity issues. Efforts to reduce heavy metal based QDs toxicity include the encapsulation in a SiO₂ shell, therefore the autofluorescent Si/SiO₂ QDs we use should join the benefits of QDs and the low toxicity of SiO₂.

The QDs were prepared by pulsed laser ablation technique. The particles were spherical and had a silicon (Si) core covered by a 1–1.5 nm thick amorphous silicon dioxide (SiO₂) surface. The lognormal size distribution estimated from transmission electron microscopy was in the range of 2–10 nm and averaged at about 5 nm. The photoluminescence emission (at room temperature) was maximum at ~690 nm (~1.8 eV).

We focused on revealing the in vivo effects induced by QDs in crucian carp, primarily in the liver. After 1, 2, and 3 weeks from intraperitoneal (IP) injection of a 2 mg/kg body weight single dose of QDs, the liver accumulation was highlighted by fluorescence imaging. After 1 week the levels of oxidative stress markers as malondialdehyde, advanced oxidation protein products and reactive protein carbonyl groups were increased, while protein sulfhydryls and reduced glutathione were reduced. Towards the second and third week, the levels of all these parameters tended to recover to values close to the control fish group (IP injected with 0.7% NaCl). This progression of these oxidative stress markers shows the early onset of redox imbalance. Notwithstanding the increased activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, counter-regulated to maintain the redox status, we suspect oxidative damage may alter protein native structure. To prevent this, heat shock proteins Hsp90, Hsp70, Hsp60 and Hsp27 were upregulated after 1 week from QDs injection, with a tendency to recover towards the end of the experiment. By contrast, Hsp60 and Hsp27 upregulation were delayed until the second week.

The protective effects of chaperones and the antioxidant enzyme up-regulation minimize the impact of Si/SiO₂ QDs accumulated in fish liver after 3 weeks from the IP injection, as demonstrated by the recovery of the oxidative stress markers levels. Our results advance the idea that silicon based QDs have moderate negative effects that yield after a period of time.

**Effect of resveratrol on nitric oxide metabolism and 3-nitrotyrosine level in heart tissues of fructose-fed rats**

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Resveratrol is a natural phytoalexin synthesized in a wide variety of plant species as a response to environmental stress. It was reported that resveratrol has many protecting properties to human health such as an antioxidant, modulator of lipoprotein metabolism, inhibitor of platelet aggregation in *in vivo* and *in vitro* studies. Fructose consumption has been known to be one of the environmental factors contributing to the development of insulin resistance, hypertension, dyslipidaemia and other abnormalities of metabolic syndrome and subsequent cardiovascular diseases. In the present study, we aimed to investigate the general metabolic alterations, nitric oxide (NO) metabolism and 3-nitrotyrosine (3-NT) levels of the heart tissue, and effects of resveratrol on these parameters in a rat model of metabolic syndrome, accomplished by high fructose feeding. For this purpose, 32 male adult Sprague-Dawley rats were randomly divided into four groups (n = 8); control, fructose, resveratrol and fructose plus resveratrol. Trans-resveratrol (10 mg/kg daily by oral gavage) and fructose (20% in drinking water) were administered for 8 weeks. Systolic blood pressures were measured by tail-cuff method. Body weights were recorded weekly and fluid intake of all groups were measured daily. After the experimental period of 8 weeks, serum lipid profile, glucose, insulin and heart tissue nitrate-nitrite (NOx), which are known to be the stable end products of NO, 3-NT, endothelial and inducible nitric oxide synthases (eNOS and iNOS, respectively) protein levels were determined. As a result, fructose consumption increased systolic blood pressure, serum triglyceride and insulin levels and insulin resistance significantly. While heart tissue 3-NT levels were elevated significantly, eNOS protein levels were found to be diminished in fructose group. Resveratrol administration prevented the increase in systolic blood pressure, and significantly increased eNOS protein to control levels in fructose plus resveratrol group. However, it did not show a protective effect on atherogenic lipid profile, insulin resistance and tissue 3-nitrotyrosine levels induced by high-fructose diet. NOx levels did not change significantly among groups and iNOS protein was not detected in any of the groups.
VLDL-cholesterol, insulin and insulin resistance. Moreover, important changes, including increase of the serum ADMA, homocysteine and NOx levels were observed following fructose treatment. Melatonin administration prevented the increase in systolic blood pressure, insulin resistance, serum insulin, ADMA and homocysteine levels induced by high-fructose diet. These results show that high-fructose consumption leads to elevated systolic blood pressure, atherogenic lipid profile, increased serum ADMA and homocysteine levels and melatonin treatment has beneficial effects on these biochemical parameters in rats.

**SW03.S13–59**

**Substrate-specific induction of epithelial-to-mesenchymal transition in Fabry disease nephropathy**

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Fabry disease is a lysosomal storage disorder caused by deficiency of α-galactosidase A (α-gal A), resulting in deposition of globotriaosylceramide (Gb3) in the vascular endothelium. Deacetylated Gb3, globotriaosylphosphogluco (Lyso-Gb3), has been suggested as a candidate biomarker, which is also accumulated in patient with Fabry disease. According to renal and cardiac pathological findings, EMT could be a possible pathogenic mechanism in Fabry disease. However, the association of EMT with Fabry disease has not been elucidated yet. We hypothesized that lyso-Gb3, rather than Gb3 in Fabry disease may be responsible for the development of EMT in kidney. In human proximal renal tubular epithelial cells (HK-2), key elements of the EMT process such as E-cadherin, N-cadherin, β-catenin and α-SMA were significantly altered in lyso-Gb3 of low concentration. Whereas, expression level of EMT marker and α-SMA in mouse renal glomerular mesangial cell (SV40MES13) significantly increased in treated Gb3. Additionally, we found that the activation of TGFβ, phospho-AKT, and PI3K were highly elevated in these cells. Our study demonstrates that accumulation of Gb3 and lysoGb3 in HK2 cell and SV40MES13 can potentiate EMT-like processes through AKT/PI3K signaling pathway. Our findings suggest that lyso-Gb3 may be a crucial role nephropathy of Fabry disease by inducing EMT and may contribute to a better understanding of the renal fibrosis.

**SW03.S13–60**

**Exposure to Si/SiO2 quantum dots induces adaptive changes in antioxidant enzyme, gelatinase activity and heat shock protein expression in carp white muscle**

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Quantum dots (QDs) are fluorescent nanoparticles increasingly used in medical imaging. A major downfall is that they’re often composed of toxic elements. This problem may be overcome by Si QDs, that theoretically possess reduced toxicity. Nevertheless particle size has been reported to account for many toxic effects caused by nanoparticles. We used autofluorescent SiO2/Si QDs with Ex375 nm/Em600 nm, and a size distribution of 2.75 nm–11.25 nm

Common carp white muscle biochemical changes were assessed after one, 2 and 3 weeks from intraperitoneal injection of 2 mg QDs/kg body weight. QDs accumulation in muscle tissue was highlighted by fluorescence microscopy. At every interval superoxide dismutase and catalase activity upregulated and peaked in the first and second week after QDs exposure, supporting a ROS generation scenario. Glutathione reductase activity also increased in the first week, suggesting that glutathione may act as a ROS scavenger by itself, since glutathione peroxidase activity remains unchanged. Protein reactive carbonyl groups and malondialdehyde levels peaked 1 week after injection with increases of 21% and 79% and returned to control values by the 3rd week. Protein thiol levels diminished only in the first week. Heat shock proteins Hsp27 and Hsp70 generic expressions increased their relative expression ratios (R) at 4.2 ± 2, 6.8 ± 0.8 and 2.4 ± 0.3 for Hsp27 and 2.4 ± 0.4, 4.2 ± 1 and 1.1 ± 0.6 for Hsp70. Hsp’s upregulation may play an important role in maintaining correct protein folding in the more oxidative intracellular environment following QDs uptake. Both MMP9 activity and transcriptional levels were reduced, showing a minimal gelatinolytic activity of 50% of control value in the second week and had overall reduced R by about 50%. MMP9 inhibition has been suggested to intervene in a reduction of the inflammatory process. MMP2 R was upregulated starting with the second week. MMP2 gelatinolytic activity is modulated indicating an inflammatory response followed by fibrosis and tissue remodeling.

Our data show that Si QDs induce a moderate oxidative stress, and the antioxidant enzymes and Hsp protective activity can counteracted it. Minimal damage to white muscles is also ensured by the reduction of MMP9 activity and MMP2 adaptive profile.

**SW03.S13–61**

**Integration of chaperone networks acting in stress defense, protein folding, trafficking and prion propagation**

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The evolutionarily conserved Hsp70 chaperones and their Hsp40 partners have been implicated in a variety of cellular processes, including nascent polypeptide folding and stress response. Together with the disaggregating chaperone Hsp104, Hsp70/40 proteins also play a crucial role in the propagation of self-perpetuating amyloid aggregates (prions) in yeast. Our recent data (Newnam et al. 2012 JMB 408: 432; Chernova et al. 2011 Mol. Cell 43: 242) indicate that the balance between the Hsp104 and Hsp70 chaperones regulates asymmetric distribution of a yeast prion in cell divisions after stress. We now demonstrate that this process is also controlled by other cellular systems involved in spatial distribution of protein aggregates and aging, including the cytoskeletal networks and sirtuin machinery. We have also shown (Kiktev et al. 2012 MCB 32: 4960) that the effects of the Hsp104/70/40 machinery on a yeast prion are regulated by the evolutionarily conserved small glutamine-rich tetratricopeptide cochaperone Sqt2, previously linked to the guided entry of tail-anchored (TA) proteins (GET) trafficking pathway. Sqt2 directly interacts with prions, chaperones and aggregates of TA proteins. Our data identify Sqt2 as an aggregate sensor targeting chaperone machinery to various types of protein aggregates, such as amyloids and by-products of malfunctioning trafficking pathways. We also demonstrate that alterations of the ribosome-associated chaperone complex promote de novo prion formation and influence effects of cytotoxic Hsps on prion propagation in the yeast cytoplasm. These results establish a connection between
the chaperone roles in nascent polypeptide folding and in formation/propagation of self-perpetuating amyloid aggregates.

**SW03.S13–62**

Transcription factor TnrA inhibits the biosynthetic activity of glutamine synthetase in *Bacillus subtilis*

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The *Bacillus subtilis* glutamine synthetase (GS) plays a dual role in a cell metabolism by functioning as a catalyst and regulator. GS catalyses the ATP-dependent synthesis of glutamine from glutamate and ammonium. Under nitrogen-rich conditions, GS becomes feedback-inhibited by high intracellular glutamine levels and then binds transcription factors GlnR and TnrA, which control the genes of nitrogen assimilation. While GS-bound TnrA is no more able to interact with DNA, GlnR-DNA binding was shown to be stimulated by GS complex formation.

The interaction of transcription factor TnrA with GS results in partial inhibition of GS activity in *vivo* and *in vitro*, while the GlnR protein does not affect enzymatic activity of GS. Furthermore, TnrA enhances the effect of feedback inhibitors, in particular, of glutamine. Addition of glutamine to wild-type and AmtB-deficient cells decreased the *in situ* activity of GS by 60 – 70%, which is almost the same level of inhibition of GS by TnrA determined *in vitro*. These data can be explained by re-localization of TnrA from GlnK to GS upon nitrogen-excess treatment and subsequent GS inhibition. Since TnrA has also the potential to interact with non-feedback inhibited GS, the interaction of TnrA with GS under nitrogen-limited growth would counteract the activity of GS. An important function of binding of TnrA to GlnK during nitrogen-limited growth could thus be prevention of unfavourable TnrA-GS interactions that could impair the activity of GS.

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**SW03.S13–63**

The eIF2alphaS51 phosphorylation pathway acts downstream of Akt and mTOR to determine cell fate in response to stress and chemotherapeutic drugs

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mRNA translation is important for cell proliferation and tumor development and represents a valid target of pharmaceutical intervention in cancer. A key step in mRNA translation involves the regulation of initiation by the eukaryotic initiation factor eIF2. Eukaryotic cells respond to various forms of stress by inducing the phosphorylation of the alpha subunit of eIF2 at serine 51 (herein referred to as eIF2alphaP), a modification that leads to a general inhibition of protein synthesis. Increased eIF2alphaP is mediated by a family of kinases consisting of PKR, PERK, GCN2 and HRI, each of which becomes activated by distinct stimuli. eIF2alphaP can act either as a promoter of cell survival but also as an inducer of cell death in response to various forms of stress. Previous work by our group demonstrated that eIF2alphaP is induced in cells subjected to genetic as well as pharmacological inhibition of the phosphoinositide-3-kinase (PI3K)-Akt pathway [Sci. Signal. 4, ra62 (2011)]. We discovered that PI3K-Akt pathway disruption leads to the activation of PERK and consequently to increased eIF2alphaP. At the molecular level, we found that Akt negatively regulates PERK by phosphorylation at threonine (T)799. We showed that Akt inhibition decreases PERK phosphorylation at T799, a process that promotes PERK activation in response to stress. To better understand the molecular events that link Akt inactivation to increased eIF2alphaP, we investigated the role of the mammalian target of rapamycin (mTOR) in the regulation of PERK-eIF2alphaP arm. We found that mTOR inhibition with either rapamycin or the new generation of catalytic inhibitors increases PERK activity and eIF2alphaP. Moreover, genetic inactivation of mTOR, Raptor or Rictor by shRNAs in mouse and human cells led to the conclusion that mTOR complex 2 is responsible for inhibition of the PERK-eIF2alphaP arm and that rapamycin increases eIF2alphaP independent of mTOR. The biological significance of our findings is highlighted by the observation that cells deficient in tuberous sclerosis complex (TSC) contain elevated levels of the PERK-eIF2alphaP arm and are more susceptible to stress than cells with intact TSC. TSC-deficient cells contain low levels of mTORC2 and Akt activity, which account for PERK activation and increased eIF2alphaP. Our work shows that eIF2alphaP is a mechanism of translational control by the PI3K-Akt-mTOR pathway, which is distinct from cap-dependent translation mediated by the eIF4F complex. Disruption of PI3K-Akt-mTOR signaling not only causes inhibition of cap-dependent translation by the inactivation of eIF4F but also results in general protein synthesis inhibition by increased eIF2alphaP. Most importantly, increased eIF2alphaP provides a cytoprotective environment which has to be eliminated in order to increase the efficacy of anti-tumor drugs targeting the PI3K-Akt-mTOR pathway.

**SW03.S13–64**

Yap1 mediates tolerance to cobalt toxicity in the yeast *Saccharomyces cerevisiae*

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Cobalt has a rare occurrence in nature, but yet may accumulate in cells to toxic levels. As a consequence, living organisms have developed sophisticated mechanisms to counteract cobalt toxicity. Here, we report that yeast cells devoid of Yap1 exhibit increased sensitivity to cobalt stress. Using genetic and biochemical approaches we have shown that cobalt excess induces oxidative stress in yeast cells and that Yap1 is required to mitigate the oxidative damages. However, when challenged with high concentrations of cobalt, yap1 mutant cells accumulate lower levels of this metal, suggesting that Yap1 is regulating cobalt cellular uptake. Accordingly, transcriptome analysis of yap1 mutant revealed novel targets of Yap1 involved in low-affinity metal uptake, such as the genes of the high affinity phosphate transporter, PHO84, and of the low affinity iron transporter, FET4. Overall our results emphasize the important role of Yap1 in mediating cobalt-induced oxidative damages and reveal new routes for cell protection provided by this regulator.

**SW03.S13–65**

Genetic systems of toxin-antitoxin as modules responsible for stress


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Toxin-antitoxin systems (TAS) are present in the genomes of the overwhelming majority of bacteria and archaea. These systems...
are involved in various cellular regulatory processes such as apoptosis, antibiotic tolerance, stress response, biofilm formation, and until now have not been investigated in Lactobacillus. Lactobacilli are important microorganisms in various activities: dairy products, meat ripening and bread but moreover are associated directly with human skin and cavities (e.g. mouth, gut or vagina). Some of them might be probiotics. Therefore, the molecular biological investigation of these bacteria seems promising. We described several TAS II type in lactobacilli. Functional role of the TAS in the microbiota gut and communication with the human body is the object of our research.

We identified six TAS (PemK1-A1n, PemK2-A2n, PemK3-RelB2n, relE1n, RelB3-RelE3n, YelM-YoeBn) in the genomes of annotated strains of L. rhamnosus and seven TAS in L. helveticus (RelBE1n, RelE2n, RelBE3n, RelBB4n, MazEF1n, MazEF2n, MazEF3n) in which toxins belong to the MazE and RelE superfamilies.

We describe the structure and differences in gene expression of a selected TAS in newly isolated strains in Russian population from gut comparing stationary and logarithmic growth phases, the influence of stress factors and RNA stability. The TAS is variably expressed, responding to physiological and stress conditions differently in related strains.

TAS heterogeneity also may be used to reveal intergenic differences between strains. Cloning the toxin genes of 4 TAS L. rhamnosus and of 4 TAS L. helveticus inhibited E. coli growth to varying degrees, thereby confirming their functionality. Cell growth arrest caused by expression of several toxin genes could be reverted by the expression of a cognate antidote. In several occurrences a single amino acid substitution in the toxin peptide rendered it non-toxic in E. coli.

SW03.S13–66
Role of GS28 on hydrogen peroxide-induced cell death in human osteoblastoma cells
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We observed the decreased expression of GS28 (golgi SNAP receptor complex 1) in ischemic hippocampus of rat brain. GS28 is involved in ER-golgi transport of proteins synthesized in ER, but almost unknown in another role. In this study, we examined the role of GS28 and its molecular mechanisms on hydrogen peroxide (H2O2)-induced cell death in human osteoblastoma cells. GS28 siRNA-transfected (K/D) cells showed significant decrease of viability in the cells treated with H2O2, compared with that of control cells. Pretreatment of GS28 K/D cells with p38 MAPK inhibitor accentuated the decrease of viability in H2O2-treated cells, which was confirmed by the viability assay in co-transfected cells with GS28 and p38 MAPK siRNA. Phosphorylation of p38 MAPK was increased both in control and GS28 K/D cells treated with H2O2. Pretreatment with ROS scavenger or iron chelator recovered the viability of the GS28 K/D cells treated with H2O2. Pretreatment of the GS28 K/D cells with pan-caspase inhibitor had no effect on viability of the cells treated with H2O2, and activation of procaspase-3 or increase of LC3-II was not observed in the cells by immunoblot analysis. These data suggest that caspase-independent cell death treated with H2O2 increases under GS28 K/D conditions. Taken together, GS28 has a protective role on H2O2-induced cell death and the expression of GS28 is dependent on p38 MAPK in human osteoblastoma cells.

SW03.S13–67
Reduction in markers of oxidative capacity during 35d spaceflight simulation is offset by concurrent resistance exercise
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This study assessed the efficacy of resistance exercise (RE) to counteract skeletal muscle metabolic perturbations induced by 5 weeks unilateral lower limb unloading (UL). Twenty-one men and women (30–56 years) were randomly assigned to either UL with (Grp UL + RE; n = 10) or without (Grp UL; n = 11) concurrent RE. Isometric RE comprised four sets of seven maximal coupled concentric-centrifugal knee extensions executed 2–3 week–1. Percutaneous biopsies were obtained from m. vastus lateralis before and after either intervention. Levels of mRNA expression of factors regulating oxidative capacity i.e., peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α) and vascular endothelial growth factor (VEGF), and glycolytic capacity i.e., phosphofructokinase (PFK), hexokinase (HK1), glycogen phosphorylase (GP), glycogen synthase (GS) and phosphorylase kinase (GPKα) were subsequently analyzed. Grp UL showed reduced (p = 0.009) PGC-1α expression, increased (p = 0.029) PFK expression, and a trend towards decreased VEGF post intervention. Grp UL + RE showed no changes (p > 0.05). These results suggest that 5 weeks unloading reduces skeletal muscle oxidative capacity, and increases glycolytic enzyme activity. More importantly, 12 bouts of high-force, low volume resistance exercise attenuated these responses. Thus, the exercise paradigm employed here, emphasizing eccentric overload, effectively counteracts unwarranted muscle metabolic alterations induced by unloading, and may therefore aid in maintaining muscle endurance, and hence astronaut health and fitness during spaceflight.

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SW03.S13–68
Protective effects of blueberry tea and blueberry wine on CCl4 induced kidney toxicity in rats
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Carbon tetrachloride (CCl4) is a well known toxicant which induces oxidative stress and causes free radical generation in many tissues such as liver, kidney which are the main routes of drug elimination. Studies showed that various herbal contents could protect organs against CCl4 induced oxidative stress by altering the levels of increased lipid peroxidation. Blueberries have among the highest antioxidant capacities of fruits and vegetables. We aimed to investigate experimentally whether blueberry tea and blueberry wine have a protective effect of CCl4 induced acute kidney damage.

Totally 32 Sprague-Dawley rats were divided into four groups. Groups were arranged as Group 1: normal diet (n = 7); Group 2: normal diet and kidney damage (n = 9); Group 3: kidney damage and blueberry tea (n = 8); Group 4: kidney damage and blueberry wine (n = 8). CCl4 was given intraperitoneally to generate kidney damage on between days 4–6. Following day of the study completion (8th day), we investigated some biochemical parame-
Abstracts

SW03.S13–69
Calcium paradox induces apoptosis in the isolated perfused vertebrate heart; involvement of p38-MAPK and calpain
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‘Calcium paradox’ is a term used to describe the deleterious effects conferred to a heart perfused with a calcium-free solution followed by repletion, including: loss of electromechanical activity, sarcomere rupture, leakage of intracellular enzymes and depletion of high-energy reserves. Of note, the signal transduction mechanisms triggered by the calcium paradox remain elusive. Therefore, in the present study, we probed into these signaling pathways, in the isolated perfused vertebrate heart. The calcium paradox was found to significantly induce activation of the MAPK family members (p43-ERK, JNKs and p38-MAPK). Lactate dehydrogenase activity as well as total protein release in the perfusate was indicative of necrotic cell death; nevertheless, we also identified poly(ADP-ribose) polymerase (PARP) fragmentation as well as upregulation of other pro-apoptotic markers. Furthermore, using MDL28170, a selective inhibitor of calpain, a role of this protease was revealed, as its inhibition almost abrogated the calcium paradox-induced apoptosis. In addition, various divalent cations such as manganese, barium and calcium were shown to exert a protective effect against the calcium paradox, leading to recovery of the amphibian heart’s mechanical activity. Interestingly, inhibition of p38-MAPK by its selective inhibitor SB203580 (10 μM), alleviated the calcium paradox conferred apoptosis. This result indicates that p38-MAPK plays a pro-apoptotic role, contributing to the resulting myocardial dysfunction and cell death in hearts subjected to the calcium paradox. Collectively, this is to our knowledge the first time that the signaling pathways stimulated by the calcium paradox have been investigated in amphibians, gaining insight in the occurring apoptosis, MAPKs and calpain involvement, as well as p38-MAPK detrimental role.

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SW03.S13–70
Curcumin-induced signal transduction pathways in H9c2 cardiac myoblasts
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Curcumin is a biologically active polyphenol, derived from the rhizomes of Curcuma longa. Its mode of action is pleiotropic and is attributed to its anti-bacterial, anti-inflammatory and anti-oxidant properties. Given the salutary role that curcumin exerts in the mammalian heart i.e. against myocardial ischemia/reperfusion injury, the present study focuses on the signaling pathways triggered by curcumin in an established experimental model such as H9c2 cardiac myoblasts.

Curcumin has been shown to interact with multiple signaling molecules, including mitogen-activated protein kinases (MAPKs). Thus, we initially examined the potential activation of two MAPK subfamilies: p38-MAPK and JNKs. Both p38-MAPK and JNKs phosphorylation levels were found to be significantly upregulated by 20 μM curcumin, for increasing time intervals. What is more, Hsp27 and ATF2 as well as cJun, established substrates of the aforementioned kinases were also shown to be phosphorylated under the interventions tested. Interestingly, probing into the effect of curcumin on H9c2 viability using MTT, we found that 20 μM curcumin for 24 h, caused a reduction in cell viability by ~20%, while 40 μM curcumin for 24 h caused a reduction by ~30%. In order to determine potential activation of the apoptotic mechanism, PARP cleavage was assessed, a known marker of apoptosis. Indeed, PARP fragmentation was detected after 6 h of incubation with 20 μM curcumin, along with cytochrome c release in the cytoplasm. PARP cleavage was found to be caspase-3-dependent and was completely abolished by 1 μM AS600125, a routinely used JNKs inhibitor. Therefore, although p38-MAPK may exert a protective role interacting with Hsp27, curcumin-induced JNKs activation seems to favor apoptosis. Additional experiments are evidently required in order to fully elucidate and clarify the exact role and action mechanism of all these mediators, in the responses conferred by curcumin.

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SW03.S13–71
Role of senescent stroma in prostate cancer progression
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Senescent fibroblasts arrest proliferation and acquire a senescence-associated secretory phenotype (SASP), leading to production of inflammatory and angiogenic cytokines. To investigate the role of the senescent stroma in the progression of prostate cancer, we focused out interest in replicative senescence (RS) and hydrogen peroxide stress-induced senescence (SIS) of fibroblasts obtained by human patients. Both RS and SIS leads to activation of SASP and secretion of IL-6, IL-8, VEGFA, MMP3, MMP2. Senescence of stromal fibroblasts favors prostate cancer aggressiveness by activation of motility through epithelial mesenchymal transition (EMT) and increasing trophic behaviour, through secretion of energy-rich compounds as lactate and ketones. Senescent fibroblasts additionally modify other components of the tumor microenvironment as they increase: (i) the recruitment of monocytes and their M2-macrophage polarization, (ii) the recruitment of bone marrow-derived endothelial precursor cells, facilitating their vasculogenic ability, and (iii) increase capillary morphogenesis, proliferation and invasion of human mature endothelial cells.

These phenomena are depended on de novo expression of miR210, an hypoxia-miRNA already involved in the ageing process, miR210 overexpression in young fibroblasts increases their senescence-associated features (SASP, DNA-damage foci) and convert them into CAF-like cells, able to promote cancer cells EMT, to support angiogenesis and to recruit endothelial precursor cells or monocytes/macrophages. Of note, all these responses...
are blocked by ablation of miR210 expression during senescence by specific AntagomiRs. Our data shed new light on the ability of senescent stroma to affect prostate cancer malignancy, sustaining the development of a favorable microenvironment for cancer progression, recruiting/organizing vessels and sustaining inflammation.

**SW03.S13–72**
The effect of combined heavy metal ions on *copA, nikA and ccdD* genes expression of metal-resistant bacilli  
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Heavy metal resistant microorganisms, thriving in environments with excessive concentrations of heavy metals, have evolved a variety of adaptation strategies. Reduced accumulation based on an active efflux of the bivalent cations is the primary resistance mechanism developed in prokaryotes. The goal of the present work was to investigate the expression of the *copA, nikA* and *ccdD* genes of metal-resistant bacilli as a metal stress response to the presence of different concentrations of a combination of Cu(II), Ni(II), Cd(II) and Zn(II) metal ions. The test organisms were *Bacillus subtilis* AG4, *Bacillus megaterium* AA1, *Geobacillus pulida* AA4 and *Clostridium saccharolyticum* LA5 strains isolated from different territories in Armenia with elevated levels of toxic metals. The resistance patterns, expressed as minimum inhibitory concentrations, were analyzed by using the agar dilution method. All isolates showed high resistance to Cu(II) and Ni(II) (up to 5 mM), but were more sensitive to Cd(II) and Zn(II) (up to 0.4 and 1 mM, respectively). PCR combined with DNA sequence analysis was used to investigate the presence of the *copA, nikA* and *ccdD* genes, which are often responsible for resistance to mentioned ions. The presence of *copA and nikA* genes was confirmed in all the strains. *ccdD* was confirmed only in *B. subtilis* AG4 and *C. saccharolyticum* LA5. Transcription analysis of selected genes in the presence of different concentrations of metal ions in the growth medium was performed by RT-qPCR. Expression of the *ccdD* gene was not detected. Highest expression *copA* and *nikA* genes was observed in the presence of a combination of Cu(II), Ni(II), Cd(II) and Zn(II) in 0.016, 0.017, 0.01 and 0.015 mM concentrations, correspondingly, i.e. at very low metal concentrations. The absence or low expression of *copA* and *nikA* genes at high metal concentrations indicates that alternative resistance mechanisms might be present.

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**SW03.S13–73**
Protective effect of natural polyphenol complex of red wine under radioinduced oxidative-nitrative stress  
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Overproduction of reactive Oxygen (ROS) and Nitrogen (RNS) species play a key role in the development of pathological changes in the organism after exposure to low doses of ionizing radiation. Radioinduced changes in the prooxidant-antioxidant balance are caused by disorders in NO synthesis and metabolism and by the unbalanced function of antioxidant enzymes.

It is known, that polyphenolic compounds of red wine exhibit pronounced antioxidant properties under development of different diseases. Therefore studying their effect on the pathological changes in the organism under low doses of radiation presents a high interest. Under the X-ray exposure (30 cGy) was observed increasing in two times the content of thiobarbituric acid reactive substances (TBARS) and nitrotyrosine-modified (NT) proteins in the cortical layer of rat kidney. At the same time, the activities of superoxide dismutase (SOD) and NO-synthase (NOS) decreased on 72 h post-irradiation, while at the earlier time points (24 and 48 h after irradiation) the activities of SOD, catalase (CT) and glutathione peroxidase (GPO) increased when compared to control. Consumption of preparation of natural polyphenolic complex of wine (NPCW) for 10 days before and during 3 days after irradiation caused an increase in antioxidant capacity of the cortical layer of rat kidney. The polyphenols reduced the content of TBARS in rat kidney after irradiation by 1.4 times compared to irradiated rats without treatment, while the activity of antioxidant enzymes and NOS as well as the content of NO stable products did not differ from controls. The consumption of NPCW also decreased the content of NT proteins, a key marker of nitrative stress, in the cortical layer of rat kidney compared to irradiated nontreated rats.

Thereby, our results show that the preparation of NPCW diminishes oxidative-nitritative stress caused by the action of low doses of ionizing radiation. Red wine polyphenols implement their radioprotective function through ROS and RNS scavenging. In this way the activities of antioxidant defense system enzymes and L-arginine/NO system components were changed and corrected after irradiation.

**SW03.S13–74**
14-3-3 positively regulates murine protein serine-threonine kinase 38 in a phosphorylation-dependent manner  
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Murine protein serine-threonine kinase 38 (MPK38)/maternal embryonic leucine zipper kinase (MELK) is a member of the family of AMP-activated protein kinase (AMPK) that respond to changes in cellular energy state. In this study, we demonstrate that 14-3-3 has a positive role in the regulation of MPK38 activity and function. The physical association of MPK38 and 14-3-3 is mediated through their C-terminal domains, MPK38 directly interacts with and phosphorylates 14-3-3 (Ser\textsuperscript{156} of 14-3-3 theta and Ser\textsuperscript{158} of 14-3-3 sigma). In addition, ectopic expression of wild-type 14-3-3, but not the 14-3-3 theta S156A (or 14-3-3 sigma S158A) mutant, dose-dependently enhances MPK38-dependent ASK1, TGF-beta, and p38 signaling by stabilizing the MPK38 protein, suggesting that 14-3-3 phosphorylation by MPK38 contributes to the stimulation of MPK38 activity and function. We also demonstrate an *in vivo* role of 14-3-3 in the regulation of MPK38 function using 14-3-3-null HCT116 cells. Together, our present results provide evidence that 14-3-3 functions as a physiological activator of MPK38 in cells.
SW03.S13–75

3-Hydroxytyrosol protects human chondrocytes against cell death and matrix degradation

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Chondrocyte death and matrix degradation represent important components in cartilage degeneration and the mechanism of cell death may play a vital role in the pathogenesis of human osteoarthritis [1]. In the last decades several scientific investigations have been performed to discover aliment or food constituents that provide health and medical benefits (‘nutraceuticals’), which might be useful for prevention or treatment of chronic degenerative- and ageing-associated diseases [2]. Therefore we have tested the ability of 3-hydroxytyrosol (HT) to protect chondrocytes from cell death in vitro. This natural compound is a polyphenol primarily released in olive mill wastewater and in olive oil.

We found that HT pre-treatment of growing cultures of C-28/I2 chondrocytes (immortalized human cell line) and primary cultures of human chondrocytes (prepared from fragments of articular cartilage obtained from adult OA patients) decreases cell death, inflammation and matrix degradation markers induced by oxidative stress and inflammation stimuli (H2O2 and the pro-apoptotic chemokine growth-related oncogene ß). In particular our results indicate that HT prevents matrix metalloproteinase-13 (MMP-13) and cyclooxygenase-2 (COX-2) increase.

Moreover preliminary results indicate that the protective effect of HT could be mediated by the modulation of several signal pathways, such as those involving the activation of c-Jun N-terminal kinase (JNK) and NAD-dependent deacetylase Sirtuin-1.

Controlled inhibition of key steps in this process could represent a novel therapeutic strategy for the treatment and cure of osteoarthritis.

References

SW03.S13–77

A novel role for the yeast bZIP transcription factor Yap4p in the regulation of sterol biosynthesis

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Yap4p from Saccharomyces cerevisiae synonymous to Cin5p and Hap1p belongs to the AP-1 bZIP transcription factors which are also present in higher eukaryotes. It appears to be involved in the protection of yeast cells against pleiotropic drugs and several forms of stress, especially osmotic stress. However, its distinct function in the cellular adaptation network and its regulation is not understood yet.

We showed previously that the quinone reductase Lot6p forms a complex with the 20S proteasome and recruits Yap4p in a redox-dependent manner. In this complex Lot6p serves as a sensor for oxidative stress and thus influences the function and stability of Yap4p. In order to investigate the role of Yap4p in oxidative stress response we performed cell viability assays with a mutant that overexpresses Yap4p under a galactose inducible promoter. In this mutant the sensitivity towards H2O2 was clearly decreased. Furthermore, we observed changes in the growth behavior and in the lipid pattern within the mutant strain. The overexpressing strain showed a significant difference in the total amount of sterol esters and an accumulation of substrates for oxygen-dependent enzymes.

Therefore our results suggest that Yap4p is involved in the response to low oxygen concentrations and this in turn influences sterol biosynthesis. To investigate the role of Yap4p in the regulation of sterol biosynthesis in more detail, we are currently analyzing the expression of Yap4p target genes. These studies will be complemented by DNA binding assays. Furthermore, we would like to investigate the potential role of Lot6p in Yap4p mediated stress response.

SW03.S13–77

Analysis of SOD2 activity and its sub-cellular localization during diclofenac-induced apoptosis in melanoma cell lines

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Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), induces apoptosis in many cell lines through an alteration of the redox state. We have investigated if the pro-apoptotic effect of diclofenac is mediated by an alteration of the redox state, focusing the attention on the involvement of SOD2, an essential mitochondrial antioxidant enzyme. The study was realised using the melanoma cell lines A2058 and SAN, and the human fibroblast immortalized cell line BJ-5ta. Analysis of propidium iodide incorporation and measurement of caspase-3 activity showed an increase of apoptosis after diclofenac treatment in melanoma cells, whereas no effect was observed in BJ-5ta; moreover, the usage of the fluorescent probes DHE and DCFH-DA allowed the demonstration that diclofenac provoked an increase of the intracellular ROS levels only in melanoma cells. Therefore, the effect of diclofenac on the protein levels and enzymatic activity of SOD2 was investigated and only in melanoma cells a significant reduction of these parameters was evident after a 48-h treatment. To investigate if the reduction of SOD2 contributed to the pro-apoptotic effect of diclofenac, the melanoma cells were transfected with siRNAs specific for the SOD2 messenger. In the presence of diclofenac, the SOD2 silencing amplified the pro-apoptotic effect of the drug, whereas no effect was observed without the drug. To highlight the mechanism by which diclofenac induces apoptosis, we evaluated the mitochondria involvement in this process, through the analysis of the sub-cellular localization of typical mitochondrial proteins. Western blotting analysis showed that in melanoma cells cytochrome c translocated from mitochondria to cytosol after diclofenac treatment. Surprisingly, the drug altered also the sub-cellular distribution of SOD2; indeed, SOD2 appeared in the cytosolic fraction at the expenses of an almost corresponding reduction of this enzyme in the mitochondrial fraction, a feature confirmed by immunofluorescence experiments. In conclusion, the diclofenac exerts its...
cytotoxic effect selectively in melanoma cells, targeting the mitochondrial; in particular, the data suggest that both reduction and cytosolic translocation of SOD2 contribute to the mitochondrial dysfunction.

**SW03.S13–78**

To controversies of treating oxidative stress-related diseases by exogenous antioxidants

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Findings on involvement of reactive oxygen and nitrogen species in a variety of pathologies led to an effort to establish their role in etiopathogenesis of those diseases. Over last decades, an increasing number of papers about oxidative alterations of essential cellular macromolecules, such as lipids, proteins and nucleic acids, involved in tissue injury has prompted the formulation of theory of free (oxygen) radical-mediated diseases. Accordingly, uncontrolled formation of free radicals (in particular those of oxygen, preferably termed as reactive oxygen species, ROS) is considered to result in oxidative stress, a cause of tissue injury, ultimately leading to the so-called free-radical diseases. However, compounds with antioxidative properties, despite their effectiveness in protecting macromolecules from their oxidative damage if evoked *in vitro*, often fail to do so *in vivo*. In fact, in a number of large clinical trials, no significant benefit was found when treating or preventing free-radical diseases by exogenous antioxidants and severe complications often appeared.

Contrary to the free-radical theory and causal role of ROS in tissue injury, the present paper deals with a novel hypothesis that ROS may similarly to other cellular and tissue mediators play an important role in intracellular and intercellular signaling. Evidence is provided that in certain cases, increased generation of ROS may occur secondarily to the tissue injury. In such situation, ROS do not play a causal role in tissue injury, but may likely be involved in signaling processes and as such in mediating tissue healing rather than injurious insult. This concept is, however, in an obvious contradiction with traditional understanding of ROS as deleterious agents. Nonetheless, one cannot exclude that under failing of autoregulatory mechanisms, ROS may be detrimental as they can contribute to evolving injury and thus aggravate the particular condition. In conclusion, issues regarding a real role of ROS in tissue injury, specifically whether ROS may serve as a common cause of a various kinds of pathologies or whether their overproduction is just a manifestation of cellular and tissue damage, remain to be elucidated.

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**SW03.S13–79**

Prokaryotic and functionally inactive eukaryotic chaperonins can induce amyloid transformation of prions

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Molecular chaperones have been shown to be involved in the processes taking place during the pathogenesis of various amyloid neurodegenerative diseases. However, contradictory literature reports suggest that different molecular chaperones can either stimulate or prevent the formation of amyloid structures from distinct amyloidogenic proteins. In the present work, we concentrated on the effects caused by two molecular chaperonins, ovine TRiC and bacterial GroEL, on the aggregation and conformational state of ovine PrP. Both chaperonins were shown to bind native PrP and to produce amyloid-like forms of ovine PrP enriched with beta-structures but, while GroEL acted in an ATP-dependent manner, TRiC was shown to cause the same effect only in the absence of Mg-ATP (i.e. in the inactive form). In the presence of chaperonin GroEL, ovine PrP was shown to form micellar particles, approximately 100–200 nm in diameter, which were observed both by dynamic light scattering assay and by electron microscopy. The content of these particles was significantly higher in the presence of Mg-ATP and, only under these conditions, GroEL produced amyloid-like species enriched with beta-structures. TRiC was shown to induce the formation of amyloid fibrils observed by electron microscopy, but only in the absence of Mg-ATP. This study suggests the important role of the cytosolic chaperonin TRiC in the propagation of amyloid structures in vivo during the development of amyloid diseases and the possible role of the bacterial chaperonin GroEL, located in the intestinal microflora, in the induction of these diseases.

The work was supported by Russian Foundation for Basic Research, project 11-04-01350-a and 12-04-91330-NNIO_a.

**SW03.S13–80**

CREB3L4 blocks adipocyte differentiation through the inhibition of C/EBPbeta transcriptional activity


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Background and aims: CREB3L4 (cAMP-responsive element-binding protein 3-like 4) belongs to basic leucine zipper (bZip)-type transcription factor family, synthesized as a membrane protein of ER-Golgi. For function, the protein is cleaved and enters the nucleus. CREB3L4 is mainly expressed in the testis of mice and responsible for spermatogenesis. In this study, we showed that CREB3L4 is expressed in the white adipose tissue and involved in the differentiation of preadipocytes into adipocytes.

Methods: The role of CREB3L4 in adipogenesis is observed by oil red O staining. The mRNA levels of typical adipocyte differentiation markers, i.e., PPARgamma, C/EBPbeta/delta and C/EBPalp, were measured by qPCR. Interaction between CREB3L4 and C/EBPbeta is confirmed by immunoprecipitation assay.

Results and Conclusion: Ectopic expression of CREB3L4 to the 3T3L1 preadipocytes results in the inhibition of differentiation of 3T3L1 preadipocytes into mature adipocytes. In contrast, CREB3L4 siRNA promoted adipogenesis of 3T3L1 preadipocytes into adipocytes. Stable expression of shRNA-CREB3L4 in 3T3L1 preadipocytes resulted in the differentiation to adipocytes by insulin. CREB3L4 interacts with C/EBPbeta and regulates transcriptional activity of C/EBPbeta. These results indicate that CREB3L4 could be a negative regulator of adipocyte differentiation.
The role of TXNIP in impaired glucose tolerance in diabetic model mice

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Thioredoxin-interacting protein (TXNIP) is upregulated in hyperglycemia causing imbalance of glucose homeostasis. However, the molecular mechanism of how TXNIP impairs glucose homeostasis in liver is unclear. Here, we show that overexpression of TXNIP upregulates gluconeogenic gene expression and impaired glucose tolerance in mice.

To investigate the role of TXNIP, mouse primary hepatocytes were incubated with/without TXNIP adenovirus (ad-TXNIP). The gluconeogenic gene expression was determined by qPCR and western blot. To observe the effect of TXNIP on the glucose homeostasis in vivo, glucose tolerance test was performed after administering Ad-TXNIP via tail vein. In order to elucidate molecular mechanism of TXNIP on the regulation of gene expression, promoter assay, promoter-cadmium interaction and chromatin immunoprecipitation studies were performed.

TXNIP gene is increased in the fatty, db/db and STZ-induced diabetic mice. Overexpression of TXNIP to the primary hepatocytes and mice resulted in an increase in the gluconeogenic gene expression. Ad-TXNIP administration to control mice exhibited impaired glucose tolerance. TXNIP is shown to interact with SHP (Small Heterodimer Partner), which is known to inhibit transcription factors upregulating gluconeogenic gene expression. This study demonstrates that TXNIP is involved in the upregulation of gluconeogenic gene expression. Increased TXNIP sequesters SHP, leading to upregulation of gluconeogenic genes and impaired glucose tolerance in mice.

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Regulation of 28 kD dehydrin content in wheat plants by 24-epibrassinolide under cadmium stress

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Dehydrins, or group II LEA (Late Embryogenesis Abundant) proteins, are known to accumulate in the seed embryos during their desiccation and also in vegetating plants in response to ABA and various stress factors that cause cell dehydration (drought, salinity, hypothermia). Plant dehydration is also caused by heavy metals (HM), among them cadmium being the most toxic. There are data on the involvement of dehydrins in HM binding and ROS neutralization, although the mechanisms responsible for regulation of dehydrin synthesis in these conditions are not clear. Earlier we have shown that pretreatment with 24-epibrassinolide (EB) significantly reduced damaging effect of cadmium on wheat plant growth. EB as well as other brassinosteroids is known to induce plants stress tolerance to various environmental stresses, including HM. Meanwhile, our data about EB-induced accumulation of TADHN dehydrin gene transcripts may indicate the involvement of dehydrins in the EB protective action. The aim of this study was to analyze changes in the content of proteins, immunopositive to dehydrin antibodies, in wheat plants in response to EB-treatment under normal conditions as well as in EB-pretreated and untreated plants under cadmium stress conditions (antibodies were kindly provided by Professor T.J. Close, USA). The results of Western blot analysis revealed dehydrins high responsiveness to 0.4 μM EB as well as to 1 mM cadmium acetate; the most susceptible to these stimuli were dehydrins of 28 kD. It is noteworthy that 28 kD dehydrin accumulation was additionally increased in the EB-pretreated seedlings exposed to cadmium. Thus, it is reasonable to assume that 28 kD dehydrin involves in the range of EB-induced protective reactions, contributing to the tolerance to subsequent toxic effect of cadmium ions in EB-pretreated plants.

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H2O2 producing/reporter system, based on D-amino acid oxidase – HyPer fusion

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Hydrogen peroxide serves as a second messenger molecule. However, elevated H2O2 production may lead to oxidative damage to cellular structures. Therefore hydrogen peroxide production for cell signaling should be strictly controlled in space and time.

Studies in redox signaling require molecular instruments for directed intracellular H2O2 generation. Yeast D-amino acid oxidase (DAAO) is an emerging tool for controlled and localized production of reactive oxygen species, allowing investigators to trigger specific signaling cascades. However neither the amount nor the diffusion rate of hydrogen peroxide produced by intracellular DAAO has been estimated yet.

We created a fusion protein of Rhodospirillum rubrum H2O2 scavenging activity in the cells on different stages of the cell cycle is yet to be determined.

As expected, cells expressing oxidase-sensor fusion protein showed faster and stronger signal than cells coexpressing the two separate proteins indicating rapid hydrogen peroxide degradation in the cells. Therefore, fusing the DAAO with potential targets of H2O2 signaling can be very efficient way to achieve fast and complete target oxidation.

Taken together, DAAO-HyPer is a useful tool allowing not only controlled H2O2 production but also immediate readout of the result.

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SW03.S13–84
Nitric oxide metabolites levels in blood in humans under exogenic acute severe normobaric hypoxia at rest
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A number of works in the last years showed nitric oxide to be involved in formation of short-term response and long-term adaptation of cells and organisms and to play significant protective role in stress, including hypoxia. These reports allow to suggest NO as one of stress-limiting systems. Taking into consideration abovementioned facts we established studying of blood levels of nitric oxide metabolites (NOx) in healthy persons during exposure to acute normobaric hypoxia.

The participants of the study were young healthy men (age 19 = 23 years, n = 16). Acute normobaric hypoxia at rest was achieved by participants breathing hypoxic gas mixture containing 8% O2 (partial pressure equivalent to 7200 m above sea levels) for 25 min. During the experiment volunteers were constantly monitored: ECG, EEG, BP, HR, gas analysis of exhaled air. Blood samples (venous blood from cubital vein) were obtained during hypoxia exposure.

Results of our study showed significant increase of blood NOx levels by 50% (p < 0.05) on 5th minute of acute hypoxia in comparison with pre-hypoxic levels. On 20th minute of acute hypoxia NOx levels in blood returned to pre-hypoxic values. Hemoglobin oxygen saturation by 25th minute of hypoxia dropped to 52.1 ± 7.1%. Analysis of lactate and pyruvate levels in venous blood revealed no significant changes under acute hypoxia. Glucose levels in participants decreased significantly by 5th minute of hypoxia but never dropped below 4.0 mM.

It is presumed that in acute normobaric hypoxia at rest energy expenditures decrease. Role of autoregulation of mitochondrial (including ‘general’) metabolic pathways is thought to increase greatly after 5–10 min of acute normobaric hypoxia. Our data suggest that evaluation of NOx values in acute normobaric hypoxia is important in assessment of acute normobaric hypoxia tolerance.

SW03.S13–85
On the role of gasotransmitters in bacterial physiology
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Until recently H2S has been known merely as a toxic gas. It is now associated with beneficial functions in mammals from vasorelaxation, cardioprotection, and neurotransmission to anti-inflammatory action in the gastrointestinal tract. The ability of H2S to function as a signaling molecule parallels the action of another established gasotransmitter, nitric oxide (NO). Like NO, H2S is produced enzymatically in various tissues. Our recent findings implicate endogenous NO and H2S as conceptually novel bacterial defenses against stress. We showed that diverse bacterial species possess dedicated NO- and H2S-producing enzymes (bNOS, CSE, CBS, 3MST) that generate these gases from L-arginine and L-cysteine, respectively. Most importantly, bacteria activate these enzymes in response to oxidative stress, antibiotic treatment, or macrophage attack, which greatly enhances bacterial survival. We showed that bacterial NO and H2S provide significant defense against antibiotics by alleviating the oxidative stress these drugs impose. Recently it was documented that many different classes of antibiotics kill bacteria by inducing oxidative damage to DNA and other macromolecules. We showed that endogenous NO and H2S protect bacteria against oxidative stress by inhibiting the Fenton reaction and by activating catalase, superoxide dismutase (SOD), and several other protective enzymes. We demonstrated that NO and H2S are dramatically induced in response to various antibiotic treatments and other insults associated with oxidative stress. Unique chemical properties of gasotransmitters define their ability to function as intercellular signaling molecules. They are small, highly perversive, and diffuse freely across membranes. Thus, they could function as short and long range ‘diffusion sensing’ signals. For example, we have shown that a small fraction of cultured bacteria that generate H2S can defend the entire population from antibiotic toxicity. Our future investigations of intracellular signaling and gene regulation mediated by NO and H2S would not only establish new paradigms of gas signaling and communication in bacteria, but it would also allow for a rational improvement of numerous existing antibiotics and the identification of novel drug targets.

SW03.S13–86
Modifications of elicitin biological activity by surface charge altering
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In plants, elicits induce a hypersensitive response and non-specific systemic acquired resistance. Because of their hydrophobic cavity, elicits exhibit an ability to transfer sterols and fatty acids between plasma membranes of plant and parasite. On the basis of their pI they are classified as either a-elicits or more necrotizing b-elicits. It has been assumed that conformational changes in the w-loop evoke by sterol binding ‘activate’ elicits. However recent results suggest that the sterol-binding ability of elicits associated with conformational changes in the w-loop, might not be principal factors in elicits biological activity and favour role of specific residues. The most probable candidates being lysine residues considering the previously observed correlation between necrotic index and pI together with a clear impact of the Lys13Val mutation in helix A on induction of a defence response in tobacco plants.

In bioreactor series of cryptogenetic mutants with changed lysine residues were produced. The significant impact of mutations on cryptogen stability measured by thermal shift assay was found. Moreover, some mutations alter the interaction of the protein with the binding site on the plasma membrane and the ability to induce a hypersensitive response and resistance. Determined results provide possibility to specifically modulate elicits biological activity through the mutation of specific residues.

SW03.S13–87
AKT/FoxO3a/Bim signaling pathway mediates ROS-induced apoptosis downstream of PTEN in selenite-treated colorectal cancer cells
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Sodium selenite is known to induce diverse stress responses in malignant cells which may lead to various type of cell death
SW03.S13–88
Carbamoylated free amino acids in uremia: HOCl generates volatile protein modifying and cytotoxic oxidant species from N-carbamoylthreonine but not threonine

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N-carbamoylation is the non-enzymatic reaction of cyanate with amino groups. Due to urea-formed cyanate in uremic patients beside carbamoylated proteins also free amino acid carbamoylation has been detected, a modification which has been linked to disturbed protein synthesis as NH2-derivatisation interferes with peptide bond formation. HOCl the product of the activated MPO:H2O2:Cl− system is known to react with the NH2-group of free amino acids to form chloramines which could exert some protective effect against protein modification and cytotoxicity induced by HOCl. As N-carbamoylation may inhibit formation of chloramines we have used N-carbamoyl-threonine as a model amino acid to study its ability to limit the reactivity of HOCl with proteins (LDL and human serum albumin) and cells (THP-1 monocytes and coronary artery endothelial cells). We also corroborated our findings in vivo by performing immunohistochemistry experiments. Overall, these results demonstrate that selenite could induce ROS-dependent inhibition of the PI3K/AKT signaling in CRC cells and xenograft tumors through FoxO3a–mediated upregulation of PTEN. Our findings help to elucidate the molecular mechanisms involved in the anti-cancer effect of selenium.

SW03.S13–89
Effects of biotic stress on the proteome of Solanum lycopersicum

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In our study, we examined the effects of biotic stress on the proteome of Solanum lycopersicum. We used three cultivars with different resistant rate to pathogen Oidium lycopersicum; highly resistant cultivar Solanum hirsutum, moderately resistant cultivar Solanum chmielewski and highly susceptible cultivar Solanum lycopersicum cv. Amateur. To induce biotic stress we inoculated fungus Oidium neolycopersici to the cultivars and 2 days after inoculation, we collected leaves from the inoculated cultivars and uninoculated control. From the leaves we isolated total protein and separated proteins by 2-D electrophoresis with isoelectric focusing in the range of pl between 4 and 7.

After the analysis of the gels in PDQuest software we found 580 protein spots on gels with inoculated cv. hirsutum and 560 spots on the gels before inoculation. Twenty were upregulated and 62 were downregulated. For cv. Chmielewski 675 spots were found before inoculation and 660 after inoculation. Fifteen were upregulated and 49 were downregulated. For cv. Amater, we found 417 spots before inoculation and 479 after inoculation. Fourteen were upregulated and 38 downregulated. The results clearly showed downregulation of large number of proteins after biotic stress. LC-MS analysis of potentially interesting proteins showed that the majority of the downregulated proteins were directly related to the photosynthetic apparatus. This is consistent with previously published studies using DNA microarrays to describe defence reactions on the model organisms Arabidopsis and Nicotiana. In these studies decrease in transcripts of photosynthetic genes in the course of the defence reactions was demonstrated.

This work was supported by the Czech Science Foundation (project P501/12/0590) and by Brno City Municipality (programme Brno Ph.D. talent). The presentation of this work was supported by the Czech Ministry of Education and European Social Fund (project CZ.1.07/2.4.00/31.0133).

SW03.S13–90
Investigation of molecular mechanisms of regulation of alanine-aminotransferase (ALT): effect of acetylcholine agonists

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ALT (EC: 2.6.1.2) is one of the key adaptive enzymes of intracellular amino acid metabolism. Effects of several cholinergic agonists and antagonists on cytosol (c-ALT) and mitochondrial (m-ALT) isozyme activities from brain and heart of white rats were investigated in vitro. Cytosol and mitochondrial fractions of the tissue homogenates were preincubated with M-agonist (arecoline) or N-agonist (nicotine) of acetylcholine at 10−3–10−10 M final concentrations for 10 min. Arecoline basically decreased the activity of c-ALT, but showed a tendency to increase m-ALT activity from rat brain. The effect of arecoline on the isozymes from rat heart was opposite: while the activity of c-ALT was increased, m-ALT one was decreased. At the same time, nicotine basically increased the activity of c-ALT, but decreased m-ALT one from brain. Oppo-
sishly, nicotine decreased the activity of c-ALT, but mainly increased the activity of m-ALT from heart. The effectors changed the initial ALT activity levels in a range of 5–30%, and the heart isozymes were found to be more sensitive than those from brain. The obtained results indicate two remarkable tendencies of the used cholinerghic agonists’ action on the ALT isozyme activities from rat brain and heart. First, while M-agonist (arecoline) showed inhibition effect, N-agonist (nicotine) basically activated the same isozyme and visa-versa for the each investigated isozyme case. Second, a direction of the agonists’ effect on the isozymes was dependent on their tissue belongings. Namely, c-ALTs (or m-ALTs) from rat brain and heart were sensitive to arecoline (or nicotine) in the opposite way: while arecoline increased c-ALT from rat heart, it inhibited c-ALT from rat brain and visa-versa. The last reverse picture was observed for the other combinations of isozyme + tissue/nicotine or arecoline. Thus, the investigated M- and N-agonists effect on c-ALT and m-ALT indicating an involvement of cholinerghic system in the regulation of the ALT isozyme activities. The direction (activation or inhibition) of the acetylcholine agonist effects are definitely determined by the localization of ALT isozymes, assuming an existence of different regulatory cholinerghic mechanisms for the investigated enzyme at tissue, cellular and molecular levels.

SW03.S13–91
Immunocytochemistry of neurotrophic factors receptors p75 and CNTF in crayfish stretch receptor organ under photooxidative stress

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Mutual support of survival between neurons and glial cells under different damaging conditions usually involves exchange of neurotrophic factors. However, signaling with neurotrophic factors, which is widespread in mammalians, was found just in few invertebrate species. The ability of NGF and CNTF to modulate effectiveness of photodynamic damage of neurons and glial cells in the crayfish stretch receptor was shown before. In this work we have done immunocytochemical analysis of distribution of p75 and CNTFR neurotrophic factor receptors in isolated crayfish stretch receptor organ – both intact and under oxidative stress. We used standard indirect immunohistochemistry protocol for whole mount crayfish stretch receptor organ preparations, which consist of sensory muscles, receptor neurons and glial cells. The primary antibodies for p75 and CNTFR were from rabbit; secondary FITC-labeled antibody was goat anti-rabbit – all commercially-available. Oxidative stress in the samples was generated photodynamically, by 30 min. irradiation with laser (633 nm, 0.3 W/cm²) after 30 min. incubation of preparations with 10⁻⁷ M of alunophthalocyanine Photosens. The well-marked immunolabeling for p75 was detected in the sensory neurons of intact preparations, which consist of sensory muscles, receptor neurons and glial cells. The primary antibodies for p75 and CNTF were from rabbit; secondary FITC-labeled antibody was goat anti-rabbit – all commercially-available. Oxidative stress in the samples was generated photodynamically, by 30 min. irradiation with laser (633 nm, 0.3 W/cm²) after 30 min. incubation of preparations with 10⁻⁷ M of alunophthalocyanine Photosens. The well-marked immunolabeling for p75 was detected in the sensory neurons of intact preparations, which consist of sensory muscles, receptor neurons and glial cells. The primary antibodies for p75 and CNTF were from rabbit; secondary FITC-labeled antibody was goat anti-rabbit – all commercially-available. Oxidative stress in the samples was generated photodynamically, by 30 min. irradiation with laser (633 nm, 0.3 W/cm²) after 30 min. incubation of preparations with 10⁻⁷ M of alunophthalocyanine Photosens. The well-marked immunolabeling for p75 was detected in the sensory neurons of intact preparations, which consist of sensory muscles, receptor neurons and glial cells. The primary antibodies for p75 and CNTF were from rabbit; secondary FITC-labeled antibody was goat anti-rabbit – all commercially-available. Oxidative stress in the samples was generated photodynamically, by 30 min. irradiation with laser (633 nm, 0.3 W/cm²) after 30 min. incubation of preparations with 10⁻⁷ M of alunophthalocyanine Photosens. The well-marked immunolabeling for p75 was detected in the sensory neurons of intact preparations, which consist of sensory muscles, receptor neurons and glial cells. 

SW03.S13–92
Crosstalk between ATF4-dependent gene regulation and mTORC1 activity during ER stress

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Stress causes transient attenuation of mRNA translation via phosphorylation of the eIF2α at Ser51 and Akt. This favors synthesis of the transcription factor ATF4. The eIF2α-P/ATF4 axis is the master regulatory mechanism for translational and transcriptional reprogramming in response to stress. Against this dogma, we observed that ER stress caused a gradual decrease of protein synthesis in Mouse Embryonic Fibroblasts (MEFs) deficient in eIF2α-P (Ser51 to Ala, A/A), with a parallel decrease of phosphorylation of mTORC1-target proteins. Translational inhibition involved only a group of mRNAs (TOP mRNAs), further confirming that ER stress in the absence of eIF2α-P decreases mTORC1 activity. In contrast, WT MEFs did not show sustained inhibition of mTORC1 activity or TOP mRNA translation during ER stress. ATF4 is known to regulate a cohort of genes including amino-acid transporters and aminoacyl-tRNA synthetases. Among them, system L-mediated Leu uptake and leucyl-tRNA synthase (LARS), are known factors that increase mTORC1 activity. We hypothesized that (i) the absence of ATF4 synthesis in A/A cells is the cause of the gradual decrease of mTORC1 activity and (ii) increased ATF4 levels in WT cells sustains mTORC1 activity. Ectopic expression of ATF4 in AA cells increased protein synthesis in control and stressed cells. ATF4-expressing AA cells had higher system L-mediated Leu uptake and increased levels of LARS. These cells during ER stress did not show inhibition of protein synthesis or decrease of mTORC1-target protein phosphorylation and TOP mRNA translation. The significance of the ATF4-mediated transcription program, leading to increased mTORC1 activity during ER stress, was investigated in mouse pancreatic b-cells (Min6), which are sensitive to ER-stress-induced apoptosis. Decreased Leu availability and inhibition of Leu uptake decreased apoptosis during ER stress. A similar response was obtained with the mTORC1 inhibitor Torin 1. Our data support an unrecognized ER stress-induced axis: ATF4-dependent induction of expression of proanabolic genes – mTORC1 activity – apoptosis. The in vivo significance of this novel stress-induced pathway is the loss of pancreatic b-cells and subsequent development of diabetes.

SW03.S13–93
Differential expression of survivin splice variants during testicular torsion and their modulation by (−)-epigallocatechin-3-gallate

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Survivin, an inhibitor of apoptosis, has a dual function in inhibiting apoptosis and regulating cell division. It is also alternatively spliced into three variants each with a suggested unique function.
We aim to evaluate the expression of survivin splice variants during testicular torsion and detorsion (TT/D) and whether their expression is affected by (-)-epigallocatechin-3-gallate (EGCG) treatment. Three mice groups were used: sham, TT/D + vehicle and TT/D treated with EGCG. The expression of survivin splice variants was measured by semi-quantitative reverse transcription-polymerase chain reaction. Histological analysis was performed to assess damage to spermatogenesis and morphometric changes in the seminiferous tubules. The effect of EGCG was monitored for all parameters measured. Survivin variant 121 was undetectable in testicular tissue. In the TT/D + vehicle group, the expression of survivin140 was markedly decreased, while survivin 40 was insignificantly lowered. Histological analyses revealed damage to spermatogenesis associated with decreased seminiferous tubular diameter, germinal epithelial cell thickness and testicular biopsy score in the TT/D + vehicle group. Changes in the measured parameters in the TT/D + vehicle animals were reversed to almost sham levels upon EGCG treatment. Our data indicate that survivin 140 is the only splice variant that might play a role in TT/D-induced spermatogenic damage and is transcriptionally regulated by EGCG treatment.

**SW03.S13–94**

**Epigenetic modification of the gene regulating oxidative stress is responsible for stress-induced behavioral change**  
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Depression is a mood disorder showing depressed mood and defective sociability, but underlying mechanism is not clearly understood. We recently reported that restraints treated for 2 h daily for consecutive 14 days (2 h–14 days) in mice produce depression-like behavior in the TST and FST, which lasted for more than a month after restraint treatment was stopped. Three weeks after the 2 h–14 days restraint, functional activity of mitochondria electron transport complex (ETC) systems in the brain was impaired. Real-time PCR analysis showed that the long-lasting change in the ETC system was correlated with persisted changes in the expression of several genes for anti-oxidative stress. ChiP assay revealed an enhancement in the acetylation and dimethylation on the histone proteins, H3 and H4, which bind to the promoter region of the BDNF and Prx3 genes. Mice injected with Lenti-shRNA-Prx3 in the hippocampus were resistant to depression-driving effects of the 2 h–14 days restraint. These results suggest that epigenetic modification of Prx3 is responsible for stress-induced behavioral change.

**SW03.S13–95**

**Effect of hypoxia and ischemia on the expression of CTGF, HGF, PLAU, PLAUR, and HBE GF genes in U87 glioma cells with IRE1 loss of function**  
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The endoplasmic reticulum stress plays an important role in the regulation of proliferative processes under hypoxic and ischemic conditions and the blockade of inositol requiring enzyme-1a (IRE-1a) function is resulted in the suppression of tumor growth. Hypoxia is responsible for regulation of numerous growth factors which control the cell proliferation and angiogenesis in malignant tumors. We studied effect of hypoxia and ischemic conditions (glucose or glutamine deprivation) on the expression of CTGF (connective tissue growth factor), HGF (hepatocyte growth factor receptor or MET), PLAU (urokinase-type plasminogen activator), PLAUR (PLAU receptor), and HBE GF (heparin-binding epidermal growth factor-like growth factor) genes which encode the synthesis of important regulators of cells proliferation and tissue regeneration in U87 glioma cells as well as its dependence from inositol requiring enzyme-1a function. It was shown that blockade of IRE-1a gene function in U87 glioma cell line is significantly increased the expression of CTGF gene, but decreased the HBE GF, HGF, PLAUR, and PLAU gene expressions. Moreover, hypoxia significantly induces the expression of CTGF gene and reduces HBE GF, HGF, and PLAU genes in control U87 glioma cells but effect of hypoxia on these gene expressions were modified in glioma cells with suppressed function of IRE1. There was observed the increase of CTGF and HBE GF gene expressions and decrease the expression of HGF and PLAUR genes in control glioma cells under glucose or glutamine deprivation conditions; however, in glioma cells with suppressed function of ERN1 we observed more strong effect of glucose deprivation condition on the expression level of HBE GF, HGF, and PLAU mRNA as well as a reversible effect on CTGF mRNA expression. At the same time, the expression of PLAUR gene was resistant to glutamine deprivation condition in control glioma cells but decreased in glioma cells with IRE1 loss of function. Results of this investigation clearly demonstrated that the expression of genes encoding growth controlling factors HBE GF, HGF, PLAUR, and PLAU is reduced by blockade of IRE1 gene function and possibly participate in the suppression of tumor growth. Moreover, most of tested genes are responsible to hypoxia and ischemic conditions in ERN1 dependent manner.

**SW03.S13–96**

**Pivotal role of Lys358 in the regulation of molecular chaperone Hsp104 ATPase activity**  
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Hsp104 yeast chaperone protein is indispensable for surviving heat shock conditions due to its ability to disentangle and reassociate aggregated proteins in ATP-dependent way. For this unique activity it co-operates with Hsp70 chaperone system. Hsp104 belongs to the AAA+ class I family, characterised by two AT-
Pase domains and ring-shaped hexameric quaternary structure. It is distinguished by long, coiled-coil M domain, absent from other AAA+ chaperones involved in proteinolysis.

In our work we postulate the importance of a certain structural aspect, namely ionic interaction network, that connects two cleafs of NBD1 ATPase domain with the M domain.

It has been recently established that the M domain is essential for interaction with Hsp70 partner. Our results suggest that interaction between M-domain residue D484 and NBD1 residue K358 is pivotal for functional cycle of Hsp104. Those residues form a part of ionic interaction network that involves also D484 residue at the other cleft of NBD1. The biochemical properties of point mutants in position 358 and 484 suggest that the role of these interactions is to couple ATPase activity with substrate translocation through the hexamer (and hence the disaggregation of substrate proteins). Reversal of either charge in those positions lead to the significant hyperactivity of Hsp104 accompanied by decreased Hsp70 requirement and loss of specificity.

ATPase activity and ATP affinity measurements, combined with structural analysis by molecular dynamics simulations lead us to the conclusion that the NBD1-M interdomain communication via K358-D484 interaction is important for allosteric regulation for the precise control of Hsp104 unfolding activity.

SW03.S13–97
Exposure of human bronchial epithelial cells to hexavalent chromium [Cr(VI)] decreases the expression of heat shock protein 90 alpha (Hsp90α) and attenuates the transient growth arrest induced by an acute cold shock

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Hexavalent chromium [Cr(VI)] has long been recognized as an occupational lung carcinogen. Awareness of this and other adverse health effects associated with exposure to Cr(VI) compounds have led to improved safety practices. On the contrary, the presence of these compounds in the environment is increasing, mostly due to industrial waste disposal, fossil fuel combustion and, possibly, tobacco smoke.

Despite numerous studies, the molecular basis of Cr(VI)-induced neoplastic transformation is still very poorly understood. Cr(VI) exposure produces several types of cellular stresses, namely oxidative and metabolic stresses, with potential relevance to carcinogenesis. Our group is currently investigating the heat shock response of human bronchial epithelial cells (BEAS-2B) to Cr(VI) exposure, as well as the impact of this response in the resistance of these cells to further stresses. Thus far, we observed Cr(VI) exposure to human bronchial epithelial cells Cr(VI) were somehow better prepared to respond to this type of shock. Experiments aimed at gaining mechanistic insight into this phenomenon, including determining the effect of Cr(VI) exposure on the protein levels of heat shock factor 1 (HSF1), are under way. This transcription factor is a major player in the heat shock response, and recent evidence points to its involvement in the regulation of several other cellular processes.

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SW03.S13–98
Desulfovibrio vulgaris Peroxide Regulon Repressor (PerR)

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Reactive oxygen species (ROS) that play an important role in the mechanisms of cell signaling and oxidations in higher organisms, are simultaneously very citotoxic. In fact they are generated by macrophages, as a weapon to eliminate pathogens. As defence cells produce protective and damage repair proteins, which are under the negative control of specific regulators. They sense the increase of ROS and transduce the signal by increasing expression of defense activities. In Bacillus subtilis the adaptive response to H2O2 seems to be under the influence of a metallo-protein called Peroxide Regulon Repressor (PerR), which is a member of the Ferric Uptake Repressor (Fur) superfamily. Like other members of Fur family, PerR is a homodimer that binds two metal ions per subunit: one zinc ion, bound by four cisteines in a site that has a structural function, and one Fe or Mn ion that is bound by three histidines and two aspartates in a regulatory site, necessary for H2O2 sensing. This function involves the oxidation of two of the Fe/Mn ligands: histidine 37 and histidine 91, but only when Fe(II) is bound to the site (Fe-PerR-Zn) [1]. Histidine oxidation seems to cause the loss of the corepressor Fe (II) and inactivation of PerR to bind DNA. This results in derepression of the genes that are under PerR influence [3].

Although long considered an obligate anaerobe, D. vulgaris can be found in some O2 exposed environments, and even though they can survive long periods of air exposure, very small amounts of O2 can affect D. vulgaris growth negatively [2]. Furthermore, studies have revealed that the transcripts of the predicted PerR regulon, in this bacterium, were upregulated during low O2 and H2O2 exposure.

A recombinant Desulfovibrio vulgaris PerR was in E.coli, and DNA binding activity tested in the presence of Fe(II) and Zn(II).


References
SW03.S13–99
Systemic oxidative stress markers in animal model for depression
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Involvement of oxidative stress (OxS) in development of major depressive disorder has recently become evident, though mechanisms behind this remain elusive. We analyzed therefore OxS pathways in rat Chronic Mild Stress (CMS) model of depression.

Rats are exposed to chronic unpredictable mild stressors and Sucrose Consumption Tests (measure of hedonic state) were performed. Some rats developed the anhedonia-like symptoms, which were evident after 4 weeks of CMS protocol. During 5th to 8th weeks of CMS anhedonic rats were treated with a selective serotonin reuptake inhibitor Escitalopram (i.p., 5 mg/kg/day). Saline injections were done to control the vehicle effect. Escitalopram treated rats were sub-divided into two groups: responders and non-responders, according to their hedonic state and compared to non-stressed rats, treated with either saline or Escitalopram. Measurement of total glutathione and malonaldehyde (MDA) in lungs, heart, skeletal muscles, liver, saphenous, mesenteric, and tail arteries were used as estimates for OxS.

In heart, glutathione was increased in CMS rats in comparison with non-stressed vehicle group. Accordingly, an estimate for free radical activity, MDA, was significantly lower in vehicle-treated anhedonic rats in comparison with both non-stressed groups and CMS non-responders group. Similarly, glutathione concentration in liver was significantly higher in vehicle-treated anhedonic group compared to all other groups. In lungs, glutathione was significantly elevated in Escitalopram-treated non-stressed rats and in CMS non-responders group (over 500 mol/ml), while it was negligible in all other experimental groups. Glutathione was lower in tail arteries from non-stressed vehicle group than in all others groups. In contrast, glutathione was significantly higher in mesenteric small arteries from the non-stressed Escitalopram-treated group than in other CMS groups. No significant changes in the OxS markers were seen in skeletal muscles and saphenous arteries. No differences between the groups in MDA were seen in any tissue with the exception of heart.

Thus, we have demonstrated tissue specific changes in the OxS markers in rats exposed to CMS and developed anhedonia-like symptoms. SSRI antidepressant treatment affected OxS pathways and related recovery was observed only in liver and lungs.

SW03.S13–100
The effect of 17beta-estradiol on GABA metabolism under impaired calcium homeostasis conditions
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GABA-shunt is the metabolic pathway involved in synthesis of γ-aminobutyric acid (GABA) from glutamate and conversion of GABA to succinate. It consists of three enzymes: glutamate decarboxylase (present in two isoforms GAD65 and GAD67), GABA-aminotransferase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH). In our earlier work we established that activity of GABA-shunt is dependent on the presence of neuron-specific plasma membrane calcium ATPases (PMCA2 and PMCA3). In present study we examined the effect of 17β-estradiol on the expression and activity of GABA-shunt enzymes under normal and impaired calcium homeostasis.

17 β-Estradiol (E2) plays an important role as transcription factor, however its receptors (ERa and ERb) are present not only in nucleus, but also in plasma membrane. This indicates on the possibility of rapid (nongenomic) action of estradiol, which can affect the metabolism not only by regulation of gene expression but also by changes in the activity of existing enzymes. It is well known that Ca2⁺ interacts with estradiol receptors. Therefore the study of the effect of E2 during disrupted Ca2⁺ homeostasis is essential.

We used stably transfected pseudoneuronal PC12 cells with suppressed expression of neuron-specific isoforms of PMCA (2 or 3). Cells were incubated with E2 for 30 min. We examined the expression of genes encoding GABA-shunt enzymes using real-time PCR analysis. Activities of GAD, GABA-T and SSADH were measured with fluorimetric and spectrophotometric methods. Our results indicate that 17β-estradiol participates in regulation of GABA-shunt. Changes are dependent on the presence of particular PMCA isoforms, specific for excitatory/secretory cells. Effect of PMCA2 suppression is opposite to the effect of PMCA3 suppression. This indicates on specific role of these isoforms on GABA metabolism in the presence of estradiol.

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SW03.S13–101
The levels of total oxidant status, total antioxidant status and homocystein in hyperhidrosis patients
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Background: The body temperature regulation mechanism is called thermoregulation. Sweating Is a natural process as well as a part of thermoregulation. Hidrosis more than required is called to be hyperhidrosis. Homocysteine is a metabolite of methionine, sulphured amino acid. Hyperhomocysteinemia in renal failure and a variety of other cases have been observed. Oxidative damage occurs when oxidant/antioxidant balance is disturbed. Oxidative stress is defined as an imbalance between the prooxidative reactive molecules and endogenous, exogenous antioxidant molecules. In our study, we aimed to determine the levels of homocysteine, TAS and TOS in hyperhidrosis cases.

Methods: Two groups were created by blood sampling from my thoracic surgery clinic patients who were admitted due to excessive sweating, hyperhidrosis, mean age 26.53 ± 10.49, 49 (27 women and 22 men), and 26 (13 women and 13 men) mean age 30.50 ± 6.26 in patients healthy volunteers. HPLC techniques were used to determine homocysteine levels in all groups. TAS and TOS commercial kit (Rel Assay Diagnostics) spectrophotometric method with Abbott Architect C8000, device was used to determine the levels of total antioxidant status and total oxidant status.

Results: Homocysteine, TAS and TOS values 15.33 ± 7.94/14.40 ± 8.04, 0.96 ± 0.20/0.91 ± 0.18, 1.42 ± 0.60/3.58 ± 2.17 (patient/control) were found in patients included in the study group and the control group, respectively. Homocysteine levels in patients and controls are close to each other. However, for patient group, TAS values are higher while TOS values are significantly lower than the control group (p < 0.05).

Conclusion: According to this study’s results, elevations in serum TAS and TOS levels may be explained by compensation...
of oxidative stress. Serum TAS and TOS levels are not diagnostic markers for hyperhidrosis. To evaluate the clinical efficiency of these markers, further studies must be performed in the surgical and medical treatment regimes of hyperhidrosis.

**SW03.S13–102**

**(-)-Epicatechin prevents palmitate-induced increase in NADPH oxidase expression and activation in the human hepatocyte cell line HepG2**

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**Background:** The incidence of metabolic syndrome (MetS) in the world’s adult population is increasing and is becoming an important health problem. MetS-associated insulin-resistance can be in part triggered by the associated increased plasma levels of free fat acid (FFAA). The activation by FFAA of NADPH oxidase (NADPHox) and the consequent production of superoxide anion and derived oxidants can in part lead to hepatic insulin resistance. Oxidants can activate kinases (JNK1,IKK) which negatively phosphorylate the insulin receptor and IRS1 leading to an impaired insulin signaling. Epidemiological studies show that the consumption of fruit and vegetables in humans decreases the risk for MetS. The flavan-3-ol (-)-epicatechin (EC) is present in large amounts in fruit and vegetables. Previously, we demonstrated that EC improves parameters of inflammation and insulin sensitivity in adipocytes.

**Objective:** The aim of our study was to evaluate the effects of EC and its metabolites on the expression and activity of NADPHox, and the consequent production of oxidants, in human hepatocytes (HepG2 cells) treated with the FFAA palmitate (Pal).

**Material and Methods:** HepG2 cells were incubated with Pal (0.25–0.75 mM), in the absence/presence of EC and its metabolites (ECM) for 24 h. The protein levels of NADPHox subunits (NOX3,p22,p47) were assessed by Western Blot. NADPHox activity was measured by lucigenin-enhanced chemiluminescence probe.

**Results:** Pal caused a dose-dependent increase in NOX3 and p22 expression in HepG2 cells. Importantly, EC and its metabolites (0.25–1 μM) treatment attenuated Pal (0.25 mM)-induced NOX3 and p22 expression. In addition, incubation with Pal also caused a significant increase in NADPHox activity, measured by both enzyme activity, and p47 translocation from the cytosol to plasma membrane. Similarly, EC and ECM (1 μM) treatment prevented Pal-mediated NADPHox activation. Moreover, Pal also caused an increase in cellular oxidants which was significantly decreased by EC and ECM.

**Conclusion:** Results show that Pal increases the production of oxidants in HepG2 through an up regulation of NOX3/p22 expression. EC and ECM, at concentrations found in plasma after consumption of EC-rich foods, prevented Pal-induced activation of NADPHox and increased oxidant production.

**SW03.S13–103**

**Natural history of kidney damage in rat in pre-transplantation period**

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One of main problems in transplantology is limitation in donor organs quantity. That is why now transplantologists focus on the usage of suboptimal donor organs and ways of their revitalization. Major factors of damage of transplant organ during transplantation are ischemia occurring after the death of donor and subsequent reperfusion in a recipient’s body. In order to understand important damaging pathways influencing on the organ in dead body we performed study of natural history of postmortem kidney as if it were before transplantologists’ assistance and initiation of conditioning procedures. We focused on the determination of so called the window of preservation of kidney in the body after the death and understanding main processes which had been initiated under ischemia condition in different time points in rat as a model organism (0, 15, 30, 45, 60, 120, 180 min and 15 h as an end-point post mortem). Specifically we analyzed in homogenized kidneys the level of caspases 3/7 activity as a mark of apoptosis, reduced and oxidized forms of glutathione as a characteristic of oxidative stress, ADP/ATP ratio as a marker of energetic state of the tissue. Also we performed quantitative analysis of gene expression in a time-scaled manner in a panel of genes involving in different pathogenic processes such as apoptosis, necroptosis, oxidative stress response as well as transcription factors involved in various pathways, components of immune response which could play an important role in subsequent damage of kidney. That allowed us to find out the window of preservation of donor organ during which injured processes were not developed to a great extent. Moreover, we have determined processes and genes which could be targets for targeted treatment for delayed and decreased injury of kidney.

**SW03.S13–104**

**Protein aggregates comprising small Heat Shock Proteins show different requirements for chaperone disaggregating machinery than other protein aggregates**

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Small heat shock proteins (sHsps) are a widespread and diverse class of molecular chaperones characterized by a low molecular mass (15–43 kDa) and a conserved stretch of approximately 100 amino acids residues. This domain displays sequence similarity to the vetebrate protein α-crystallin and therefore is called the α-crystallin domain. E. coli possesses two sHsp paralogues: Ibpa and Ibpb. It was shown by our group, that unlike other chaperone proteins, they do not disaggregate aggregated proteins, nor do they protect them from aggregation. Rather, they bind to aggregating proteins, forming with them small, soluble complexes. These complexes can subsequently be renaturated by bacterial Hsp100/Hsp70 systems at a much higher rate and efficiency that...
regular protein aggregates. However, for this situation to take place, the strong interaction between sHsp and substrate must be broken. Here, we analyze the Hsp100’s and Hsp70’s implication in abolishing sHsp-substrate interactions and renaturation of processed complexes.

**SW03.S13–106**

**Biological effects of weak magnetic fields: model study of plausible mechanisms on the developing rat myocytes**

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High penetration ability makes weak (μtesla range) static magnetic fields WSMF very interesting for biology. Earlier, we showed that biochemical manifestations of WSMF action on HeLa and VH-10 cells resemble stress reaction. Here we studied WSMF effects on the proliferation of satellite myocytes. Cells from newborn rat limb muscles were cultured on glass coated with poly-D-lysine in CO₂-incubator. DMEM medium contained antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) and fetal calf serum (10% and 2%). WSMF was created by permanent magnets placed in a shielding chamber to reduce the effect of external fields. The chamber made of nanocrystalline material provided 160-fold attenuation of the geomagnetic field. Contracting myotubes were monitored with a digital camera and inverted microscope. To measure intracellular [Ca²⁺], we incubated cells in Ringer solution with fluorochrome. Fura-2AM (10 μM) was used as fluorescent probe. Membrane depolarization was achieved by increasing K⁺ concentration (120 mM). To study the activity of ryanodine receptors (RyR) we used 4-chloro-m-cresol (1.0 mM), RyR blocker dantrolene (30–90 μM) and nifedipine (20–50 μM) as L-type Ca²⁺-channel blockers. In the experiments with calcium-free medium we added EGTA (1 mM). We showed that WSMF, only 2–3 times exceeding the geomagnetic background, accelerates the development of the cells leading to the formation of multincule hypertrophic myotubes. Exposure of the cells to such a field results in 1.5/3.5-fold increase in the intracellular calcium concentration ([Ca²⁺]) due to Ca²⁺ release from the sarcoplasmic reticulum through the RyR. We found that fully differentiated myotubes were less sensitive to WSMF, manifesting a gradual decrease in contractions frequency, whereas myotubes, in which electromechanical coupling was forming, dramatically reduced contraction frequency during the first minutes of their exposure to WSMF. Thus, WSMF was shown to have impact on the proliferation and differentiation of cultured myocytes and contractile activity of myotubes, apparently realized via intracellular Ca²⁺-signaling system. According to our data WSMF can differently affect phases of muscle fiber formation and may be considered as plausible means for correction of impaired myogenesis.

**SW03.S13–107**

**Haponin – new regulator of cellular response to oxidative stress**

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To study the functions of the recently discovered protein haponin we created panel of genetically modified cell lines with altered levels of haponin expression [1]. Using laser confocal microscopy of cells overexpressing fusion protein GFP-haponin and immunofluorescence staining of endogenous haponin we showed that haponin, having potential nuclear localization sites in its amino acid sequence, predominantly located in the cell nucleus [2]. Also, using cells overexpressing wild-type haponin and the cells in which the expression level of haponin was reduced by siRNA, it was found that under conditions of oxidative stress cell death directly correlated with the amount of haponin in the cell. The data on the involvement of haponin in molecular mechanisms of cellular response to the oxidative stress in combination with the previously demonstrated ability of haponin to bind GAPDH [3] suggest that haponin appears to play a role in GAPDH-mediated apoptosis induced by oxidative stress [4].
SW03.S13–108
Modulation of fatty acid uncoupling action in liver mitochondria under condition of oxidative stress

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Free long-chain fatty acids play an important role in oxidative metabolism as natural uncouplers of oxidative phosphorylation under various physiological and pathological conditions. In the absence of calcium ions in the incubation medium, the protonophore mechanism of uncoupling activity of fatty acids is realized with involvement of ADP/ATP- and aspartate/glutamate antiporters. Mitochondria are extremely sensitive to oxidative stress caused by reactive oxygen species (ROS) resulting in lipid peroxidation, DNA mutations and protein oxidation. It was suggested that assumed that proton permeability can be enhanced after modification of ADP/ATP-antiporter by a lipid peroxidation products. It is tentative to suppose, that aspartate/glutamate antiporter properties also change. One result of these changes may be in removal of ability the ligands of both antiporters to suppress uncoupling efficiency of fatty acids. Organic hydroperoxides, i.e., TBH, are widely used as inducers of the mitochondrial permeability transition. However, the effect of these substances on protonophore uncoupling activity of fatty acids in mitochondria was not studied yet. It is also challenging to explore the role of free radical reactions in protonophore uncoupling activity of fatty acids. As an antioxidant water-soluble analogue of tocopherol – Trolox was used. The effect of TBH and Trolox on protonophore uncoupling activity of palmitate in the presence and in the absence of ADP/ATP-antiporter ligands carbonylcytrate and ADP and of aspartate/glutamate antiporter substrate aspartate was studied. The received data demonstrate that activation of free radical processes in liver mitochondria can result in increase of protonophore uncoupling activity of palmitate by removal of recoupling effects of ADP and aspartate.

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SW03.S13–109
Oxidative and reductive routes investigation in thermophilic bacilli strain Geobacillus toebii ArzA-8 under influence of oxidizing and reducing reagents

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The ability of an organism to carry out oxidation-reduction reactions depends on the oxidation-reduction state of the environment, or its oxidation-reduction potential (ORP) [1]. The strain Geobacillus toebii ArzA-8 has been isolated from Arzakan (Armenia) geothermal mineral spring. Bacterial aerobic growth (OD), ORP kinetics and medium pH were investigated upon the absence and the presence of different concentrations of glucose (1–22 mM) at 55°C and pH 7.5. Upon absence of glucose growth medium alkalization was observed: after 24 h growth pH was 8.4. However, in the presence of 5, 11, 22 mM glucose medium acidification effect was determined at the end of log growth phase: growth medium pH has dropped up to 5.9, 5.7, 5.4, respectively. Maximal growth was in the medium without glucose supplementation (OD = 0.9) and with 5 mM glucose, however, at the beginning of stationary phase in the medium with glucose drop of OD was observed followed by medium acidification. ORP fall was observed during bacterial growth log phase measured both titanium-silicate (Eh) and platinum (Eh’) electrodes: Eh dropped down to negative values of ~150 mV and Eh’ ~350 mV without and ~100 mV and ~300 mV with glucose supplementation. 1 mM oxidant potassium ferricyanide has had negligible effect while 2 mM inhibited bacterial growth ~1.25 fold. Reducing agent DL-dithiothreitol (3 mM) repressed bacterial growth at the stationary phase but stimulated ~1.3-fold at the beginning of log growth phase.

The findings indicate that oxidative conditions are essential for G. toebii ArzA-8 growth and probably this bacterium has particular mechanisms to overcome ORP stress.

Reference

SW03.S13–110
Some like it dry: hsp in the sleeping chironomid and their role in the complete desiccation resistance

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The larvae of the sleeping chironomid Polypedilum vanderplanki, inhabiting temporary water pools in semi-arid regions of Africa, are able to revive after decades of complete desiccation and rapidly restore metabolism. In contrast to other insects adapted to the water deficit, the larvae do not prevent complete dehydration of the body, but, instead, enter ametabolic state called anhydrobiosis, replacing water with trehalose and other molecular protectants. We have first analyzed the expression of genes encoding and one heat shock factor (Pv-hsf1) in dehydrating, rehydrating, and heat-shocked larvae. All examined genes were significantly up-regulated in the larvae upon dehydration and several patterns of expression were detected. Among them, two small alpha-crystallin heat shock proteins (sHSP) transcripts were accumulated in
the desiccated larvae, but showed different expression profiles. In order to get further insights on the evolution of hsp in the sleeping chironomid and their roles in anhydrobiosis, we conducted whole genome comparative analysis of hsp groups in *P. Vanderplanki* and desiccation-sensitive species from the same midge genus *P. nubifer*. While the main groups and total number of hsp gene members were similar in two species, we have observed significant differences in expression of orthologous chaperons in response to desiccation. Our data indicate that evolutionary adaptation to extreme desiccation in the sleeping chironomid was associated with formation the hsp clusters specific in their expression to the desiccation stress. We also discuss possibility of existing of the desiccation-specific gene expression control elements in the genome of the sleeping chironomid.

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**SW03.S13–111**

*Induction of reactive oxygen and nitrogen species at different stages of the cell cycle and after exposure of human K562 and HL60 cells to ionizing radiation*

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Increased levels of free radicals appear in diverse cellular processes and are candidates for mediating long-lasting effects, including genetic instability, in cells exposed to genotoxic factors such as ionizing radiation. To better understand the processes leading to increased levels of specific reactive oxygen species (ROS) we assayed their levels, the mitochondrial membrane potential and mass, and the frequency of DNA strand breaks, apoptosis and necrosis in human leukemic cells (K562 and HL60) after X-irradiation with 4 Gy and in cells artificially synchronized by thymidine block (an inhibition of DNA synthesis attributable to imbalance of the nucleotide pool). An increase in intracellular ROS level was observed immediately, and about 24 h after irradiation, when cells were arrested in G2 phase and a second increase of ROS was accompanied by an increased level of nitrogen oxide and increases in mitochondrial potential and mass in both cell types. The second peak of ROS level was partially inhibited by rotenone, an inhibitor of mitochondrial complex I, in K562 but not in HL60 cells suggesting that the sources of ROS differed in the two cell types. The frequency of DNA breaks showed kinetics similar to ROS levels, with a sharp peak immediately after irradiation and a second increase 24 and 48 h later which was significantly higher in K562 cells. Both cell types showed a significant increase in the frequency of apoptotic and necrotic cells 24 h after irradiation, necrosis being more frequent than apoptosis in HL60 cells. Our results show that ionizing radiation activates cellular processes which produce long-lasting ROS and RNS radicals, which may have different sources in different cell types and could participate in cellular signaling networks important for radiosensitivity and for the mode of cell death.

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**SW03.S13–112**

*Mitochondrial carnitine/acylcarnitine transporter under oxidative stress: mechanism of inhibition by H2O2*

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H2O2 interacts with proteins causing alteration of activity. At concentrations below 50 μM, H2O2 also plays signalling roles. It is produced in cells as a by-product of respiration in mitochondria [1]. Thus, the primary targets of this compound are mitochondrial proteins. Since H2O2 is reactive towards thiol groups (SH), proteins with exposed Cys residues will be preferred targets. The mitochondrial carnitine/acylcarnitine transporter (CACT) which has a pivotal role in the β-oxidation of fatty acids, possesses six Cys residues. Some of these residues are exposed towards the extra-membrane space, thus being a potential target of H2O2. CACT, translocates acylcarnitines into the matrix in exchange with carnitine. The transporter has been characterized in proteoliposomes and structural data has also been revealed by homology modelling and site-directed mutagenesis [2].

The effect of H2O2 has been studied in this work on the Wild Type and Cys mutants of CACT in the proteoliposome experimental system by measuring the effects of the compound on the transport monitored as δ-harnitine/carnitine antiport. H2O2 inhibits WT CACT with an IC50 of 170 μM. The inhibition is reversed by DTE, N-acetylcysteine and Cysteine. At longer exposure times, H2O2 causes irreversible inactivation forming sulphonic species. Inhibition experiments on Cys mutants of CACT demonstrate that C136 and C155 are the targets of H2O2. The compound causes after short time treatment, the formation of a reversible disulphide between C136 and C155. This mechanism will have pathophysiological implications modulating the activity of CACT in response to variations of H2O2 levels. Thus, at lower concentrations H2O2 may tune the β-oxidation flux. At higher concentrations the β-oxidation should be blocked. This regulatory effect could contribute in switching the metabolism from lipidic to glucidic ones, under oxidative stress.

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**SW03.S13–113**

*Analysis of the cancer cell lines and the stress protein response to Annona muricata*

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Graviola from Annonaceae is known for the high content of bioactive substances. The ethanol extracts there are particularly acetogenins, showing spectrum of antibiotic, antiviral, antiulcerogenic, and antihyperglycemic effects. It was found that
acogenines are potent inhibitors of complex I NADH-ubiquinone reductase in mitochondria and specific treatment with annocin caused concentration-dependent decrease in ATP. Because of the lipophilic nature, acogenines are more toxic than rotenone to mesencephalic neurons and cause symptoms of atypical parkinsonism. Conversely, the ability of induction the reactive oxygen species was found to be effective against many cancer cell lines.

Currently available to users are not extracts, but capsules with dried leaves. We followed the effect of such supplement on different types of cancer cell lines (Jurkatt, HeLa, CEM, MCF-7, A-549 and Caco2) in concentration-dependent manner. Concentration of 31.2 μg/ml caused reduced survival of all types of cells lines. Increased concentration in the medium to 62.5 μg/ml caused decrease in viability of HeLa, MCF-7 and A-549 cells under 50%. Consequently, activities of superoxide dismutase and levels of reduced glutathione at the indicated concentrations were measured. Interestingly, the ability to scavenge radicals remained almost unchanged. As in the case of the hydroxyl radical, the values fluctuated slightly around 40%, but the antioxidant enzyme activity with increasing concentration significantly decreased. At a concentration of <62.5 μg/ml levels of reduced glutathione were several times higher than in the control group, indicating the protective effect. Vice versa, the increase in graviola concentration over mentioned value, resulted in almost complete depleted levels of reduced glutathione when compared to control.

The preparation of dried leaves demonstrated cytotoxic effects and affected redox state of cells. However, it seems that the difference in doses that would only lead to the activation of cellular stress response pathways without adversely affecting their function is small. For preventive use dosage will probably need to take into account the weight of the body to achieve the desired effect.

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**SW03.S13–114**

**Biological activities of selected ferrocenyl chalcones in vitro**

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Ferrocene as a super aromatic compound shows interesting redox properties, chemical stability, and in course of several of its derivatives also relative safety in many mammalian species. Chalcone as the structure template allows substitution of aromatic rings by ferrocene, which location influence further physicochemical properties of the compound. When ferrocene is ring B, free radical generation should be greater, since ferrocene is more readily oxidized to ferricenium. The series A compounds were characterized by more resistant to oxidation, generally lower lipophilic, but more polarized carbonyl bonds.

We followed hydroxyl radical quenching properties of (E)-3-(ferrocenemethylene)-4-chromonanone, (E)-2-(ferrocenemethylene)cylohexanone, 1,1′-bis [(E)-(2-indanonyl)-methylidenef]ferrocene, 1,1′-bis [l(E)-(2-tetralonyl)-methylidenef]ferrocene at two concentrations, which most influenced the survival of cancer cell lines in vitro. The highest radical quenching activity reached 25% at a concentration of 0.1 μg/ml in the first compound. At a concentration of 0.01 μg/ml activity generally declined to 10% in all but to 2% for the second-mentioned. Common mechanisms involving the generation of hydroxyl but first superoxide radicals are primarily associated with mitochondria.

The activity of superoxide dismutase decreased in mitochondria with higher concentration used, even significantly by the treatment with the first three compounds. Reduced glutathione levels remained significantly higher when compared to control. We assessed decrease only in the case of a third structure. Lower concentration resulted in an increase in the enzyme activities. Reduced glutathione levels remained higher only for the third derivative, among others led to its significant decline. For monitoring the level of reduced glutathione mitochondria is relatively suitable object because it does not synthesize itself. Again, the most significantly decrease in the third ferrocene derivative.

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**SW03.S13–115**

**Integrin alpha-2/beta-1 opposes senescence in human melanoma cells**

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The functions of integrins in cell senescence are poorly characterized. Here we explored the impact of a2b1 integrin silencing on cell senescence in two lines of human melanoma cells. Senescence was evaluated by percentage of cells producing increased amount of beta-galactosidase. Depletion of a2b1 was established using lentivirus based transduction of cells with the alpha-2 specific shRNA. Senescence of melanoma cells was shown to be strongly enhanced upon cytostatic-induced up-regulation of p53. In intact cells, down-regulation of a2b1 had no visible effect on senescence. In contrast, cells with the up-regulated p53 expression demonstrated 2-fold increase of senescence in response to depletion of a2b1. Silencing of a2b1 stimulated CDK inhibitor p21 while inhibited the activity of kinases FAK and Erk as well as decreased the expression of collagenase MMP-9. The data reported provide the first evidence for integrin a2b1 to implicate in senescence in tumor cells.

**SW03.S13–116**

**Oxidative stress-induced premature senescence of human endometrium-derived mesenchymal stem cells**

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Mesenchymal stem cells with multilineage differentiation capacity are attractive sources for cell-based therapy. The specific responses of these cells to oxidative stress may play a crucial role in regulation tissue homeostasis as well as regeneration of organs after oxidative injury. The responses of human endometrium-derived mesenchymal stem cells (hMSECS) to oxidative stress remain still unknown. Here, the impact of exogenous hydrogen peroxide (H2O2) on cell viability evaluated by MTT assay was examined. hMSECS were found to be much more resistant to H2O2 compared with human diploid fibroblasts (HDF). LD50 values correspond to 15–17.5 pmol/cell and 3.5–4.0 pmol/cell for hMSECS and HDF, respectively. In this study, a hypothesis whether hMSECS after exposure to sublethal doses of H2O2 (200 μM) may undergo the stress-induced premature senescence (SIPS) was tested. hMSECS subjected to oxidative stress for 1 h manifested features of SIPS: increased SA-β-Gal activity, cell hypertrophy, irreversible cell cycle arrest and loss of proliferative potential. Moreover, the lack of DNA replication detected by im-
munostaining for Ki-67 protein, substantial up-regulation of cyclin-dependent kinase inhibitor p21, as well as significantly decreased level of cyclin D1 protein were revealed. In addition, essential proteins that in DNA damage response, including protein kinase ATM and its downstream target p53, adaptor protein 53BP1, and chromatin modifier H2AX were found to be activated in hMESC's subjected to sublethal oxidative stress. These nuclear events were accompanied with the rapid hypophosphorylation of retinoblastoma protein (pRb). The results obtained demonstrate for the first time that hMESC's in response to sublethal oxidative stress undergo premature senescence, and suggest that ATM/p53/p21 signaling pathway plays a crucial role in development of oxidative stress-induced senescence program in human mesenchymal stem cells derived from endometrium.

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**SW03.S13–117**

**Mitochondria-targeted compounds decrease TNF-α-dependent endothelium activation**

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Inflammation is an important component of the innate immunity. The increased level of pro-inflammatory cytokines, such as TNF-α is usually observed in chronic and acute inflammation. The cytokines increase leukocyte adhesion and vascular permeability. One of the key factors mediating intercellular adhesion is ICAM1. Overexpression of ICAM1 is observed in many pathological conditions including autoimmune diseases, vascular dysfunctions and aging.

We have previously shown that mitochondria-targeted antioxidant SkQ1 is able to increase the wound healing speed in old animals. In the current work we aimed to elucidate the mechanism underlying SkQ1 activities on endothelium cells, notably ICAM1 expression.

Incubation of endothelial cells with picomolar concentrations of SkQ1, or with its derivate without antioxidant moiety C12TPP (C12-triphenilphosphonium), or with classical mitochondrial uncoupler DNP (2, 4-dinitrophenol) led to decreased expression of level of ICAM1 mRNA. These compounds also prevented TNF-α-induced increase in ICAM1 expression as well as secretion of proinflammatory cytokines IL-6 and IL-8. Since SkQ1 and C12TPP were previously shown to have mild uncoupling activity, we assume that mitochondrial uncoupling leads to downregulation of cellular adhesion molecules expression.

MAPK- and NF-kB-signaling pathways are the main mechanisms regulating TNF-α-related ICAM1 expression. Since MAPK-signaling pathway didn’t have significant impact on the ICAM1 expression under our experimental conditions we focused on the NF-kB-signaling pathway. We have shown SkQ1 and other uncouplers to inhibit various stages of NF-kB-signaling pathway including IkBa phosphorylation and p65 translocation into the nucleus.

In vivo experiments also proved anti-inflammatory activity of SkQ1. The level of ICAM1 mRNA in aortas of SkQ1-treated old mice was significantly lower than the level of ICAM1 mRNA in the aortas of non-treated mice.

The obtained results proved that mitochondrial uncouplers downregulate ICAM1 expression via NF-kB-signaling pathway assuming mitochondria to play important role in the inflammatory processes thus opening new possibilities for the development of next generation drugs against various endothelial dysfunctions.

**SW03.S13–118**

**The influence of VEGF-D on redox homeostasis of human endothelial cells**

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The member of VEGF family; vascular endothelial growth factor-D (VEGF-D) has been shown to promote lymphangiogenesis and also, in some extend, angiogenesis in *in vitro* and *in vivo* models. Angiogenesis induced by VEGF-D is NO production-dependent.

VEGF-D was previously reported by us to induce proangiogenic/migrative phenotype in HUVEC (Papiewska-Pajak I, Boncela J, Przygodzka P, Cierniewski CS. Autocrine effects of VEGF-D on endothelial cells after transduction with AD-VEGF-D(DeltaC). Exp Cell Res. 2010;316(6):907–14). In presented study we analyzed whether VEGF-D is implicated in regulation of redox balance in endothelial cells. Regulation of activity of cellular sources of free radicals has a critical importance for the physiology and pathology of endothelial cells.

Based on western blot results we postulate that VEGF-D upregulates expression of selected elements of antioxidant barrier: superoxide dismutase 2 (SOD2), catalase, small antioxidant enzymes that are also involved in control of cytokine-induced peroxide level – peroxiredoxin 2, 3 and 6, as well as CLIC1 and 4 – proteins which undergo a redox-controlled structural transition. On the other hand, HUVECs treated with VEGF-D revealed considerably higher ROS production, nevertheless elevated ROS amount didn’t exert cytotoxic effect on viability of ECs, suggesting rather their role as messengers in signal transduction. Thiol group content (in experimental conditions), assessed by monobromobine oxidation, was not affected by growth factor-treatment.

We also observed antiapoptotic/cytoprotective influence of VEGF-D against H2O2/BHOH-induced oxidative stress conditions (reazurin reduction assay).

Taken together, we suggest the stimulatory effect of VEGF-D on redox homeostasis of EC shown in ROS production, expression level of selected antioxidant enzymes, which may affect basic functions of endothelial cells such as angiogenesis.

**SW03.S13–119**

**VEGF and PAI-1 in endothelial cells apoptosis: balance between survival and death signals**

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Programmable cell death or apoptosis of vascular cells is an important process that occurs during blood vessel remodeling under both physiological and pathological conditions. It is also an important determinant in the fate of tumor growth and the formation of an atherosclerotic plaque. Components of the plasminogen activator system are implicated in these processes. Our recent data emerged the tenet that PAI-1 may affect processes leading to apoptosis in endothelial cells. We showed that PAI-1 directly interacts with proteasome and inhibits its function (Boncela et al., JBC, 2011). Therefore, the present study is designed to corroborate the ability of PAI-1 to interact with proteasome may...
provide a novel mechanism for the intracellular PAI-1 – dependent regulation of apoptosis in the context of angiogenesis process and vascular remodeling.

To test our hypothesis we employed truncated PAI-1 cDNA’s (without signal sequence) in pcDNA vector to obtain endothelial cells overexpressing PAI-1 mostly accumulated within the cells. Using this cellular model we demonstrate that PAI-1 abolishes prosurvival action of VEGF in cell death induced by serum withdrawal, mainly by inhibition of expression of inhibitors of apoptosis (members of Bcl-2 family). Accumulation of PAI-1 did not affect the level of pro- and anti-apoptotic factors in quiescent endothelial cells however. Based on the observations that VEGF as a pivotal survival factor activate NFkB signaling pathway we conclude that PAI-1 via proteasome interaction blocks this pathway and modulate endothelial cells survival.

To sum up, the inhibition of secretion of PAI-1 and its accumulation in endothelial cells, which is not excluded in vivo, can modify their proliferative balance and induce their apoptosis.

**SW03.S13–120**

**Methanol controls plant bacteria-host interactions**

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Plants generate a complex defence reaction in response to pathogen attacks that includes the emission of volatile organic compounds. Recently, we showed that methanol emitted by injured plants causes a phenotypically inconspicuous priming reaction in neighbouring plants i.e., the preparation of a plant receiver for a potential bacterial attack. The so-called methanol-inducible genes (MIGs) play a key role in such priming. Included among these genes are β-1,3-glucanase (BG), NCAPP (non-cell-autonomous pathway protein) and the previously unidentified gene MIG-21. In this study, we performed a search for a natural system to study the role of the intercellular transport of macromolecules in plant antibacterial immunity. Tobacco ‘sink’ and ‘source’ leaves fulfilled our requirements. The main difference between such leaves is the degree of plasmodesma ‘openness’. In intact tobacco plants, only small upper leaves, also known as ‘sink’ leaves, which are the acceptors of photoassimilates, are characterised by the presence of open plasmodesmata and intense intercellular transport. Mature, large tobacco leaves, i.e., ‘source’ leaves, are the donors of photoassimilates. We compared the ability of tobacco ‘sink’ and ‘source’ leaves to support Agrobacterium tumefaciens growth and found no difference between them, although the leaves showed a significant difference in the transcriptional activity of some MIGs, such as BG and MIG-21. However, methanol dramatically enhanced the antibacterial immunity of the ‘sink’ and ‘source’ leaves, and this enhancement was accompanied by activation of the transcriptional activity of the NCAPP gene. We concluded that the elevated level of NCAPP and not the degree of open plasmodesmata plays a key role in the increase in plant antibacterial immunity. Although bacteria are mainly extracellular pathogens that do not need to transfer their genetic material into plant cells, the nucleocytoplasmic transport of their protein virulence factors is an important stage of plant cell colonisation. We found that NCAPP can suppress the nucleocytoplasmic transport of bacterial virulence factors. We concluded that methanol controls plant immunity by affecting the nucleocytoplasmic transport and intercellular transport of macromolecules.

**SW03.S13–121**

**Effects of exercise and caloric restriction on metabolic syndrome induced hepatic oxidative stress in rats**


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In the pathogenesis of the metabolic syndrome, an increase of oxidative stress may play an important role which is closely linked with insulin resistance, endothelial dysfunction, and chronic inflammation. The aim of this study was to assess the effects of exercises and caloric restriction on some hepatic oxidative stress parameters in rat with metabolic syndrome. Fifty-six male Sprague-Dawley rats, weighing 250–350 g, were divided into five groups: control, metabolic syndrome, metabolic syndrome with exercise, metabolic syndrome with caloric restriction and metabolic syndrome with exercise and caloric restriction. To induce metabolic syndrome 10% fructose solution was given to rats in their drinking water for 3 months. Exercise and caloric restriction were applied to the related groups for 3 weeks after the induction of metabolic syndrome. In all rats, liver reduced glutathione and lipid peroxidation levels, superoxide dismutase, catalase and tissue factor activities were measured and results were discussed.

**SW03.S13–122**

**The effects of thymol and thyme oil on differentiated PC12 cells with downregulated Mgst1**

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Protection against oxidative damage is one of the most widely described attributes of polyphenols. Thymol, a dietary monoterpene phenol derivative is found in the oils of thyme and many plants, and possesses antioxidant and lipid antiperoxidative properties. However, some polyphenol supplements could be detrimental, since they could exhibit the hormetic effects: at a low-dose stimulation and at a high-dose inhibition. Microsomal glutathione transferase 1 (Mgst1) plays a specific role in protection of the cells and displays both, glutathione transferase and peroxidase activities that distinguishes this enzyme from other glutathione transferases. However, less information is available on Mgst1 functioning in neuronal tissue. This study was undertaken to evaluate the effect of 24 h incubation with selected concentrations of thymol and thyme oil (100 μM and 400 μM) on differentiated PC12 cell line, a widely accepted neuronal model system. Based on the reports showing a decrease in Mgst1 expression during aging, we also used stable transfected PC12 cell line with downregulated Mgst1 (PC12_M). Whereas the antioxidant potency of thymol and thyme oil increased in control cells, Mgst1 reduction induced necrosis with concomitantly enlarged TBARS content, and these effects were augmented by incubation with higher concentration of thyme oil. Accompanying decrease in the expression level of glutathione metabolizing enzymes: γ-glutamylcysteine ligase, glutathione synthetase and glutathione reductase, as well lowered total level of glutathione and ATP in PC12_M cells indicated on toxic effect of 400 μM thyme oil. Since the increasing interest in natural dietary components has
focused attention on plants used as a rich source of bioactive phytochemicals, our results suggest that the thyme oil should be used with caution particularly by elderly people.

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**SW03.S13–123**

**The influence of long-term administration of diet enriched with the mixture of antioxidants, probiotics and polyunsaturated fatty acid on the antioxidative status in rats spleen**

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In physiological condition body cells produce a small amount of reactive oxygen species (ROS) which influence biological processes. At high concentration ROS are associated with different steps of carcinogenesis. The defense mechanism that can protect against oxidative stress is represented by inter alia: superoxide dismutases (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-R).

The aim of the study was to evaluate the antioxidative status of spleen in rats fed diet supplemented with the mixture of different sources of biologically active compounds (polyphenols, omega-6 and omega-3 fatty acids, beta-carotene, probiotics and prebiotics). The experiment was conducted on 54 Sprague-Dawley rats which were randomly divided into two experimental groups (fed with control or supplemented diet, both n=27). Control diet was a semi-synthetic diet formulated according to the nutritional requirements for laboratory animals [1]. The supplemented diet was enriched with above mentioned bioactive substances. After 3, 6 and 12 months of experiment animals were sacrificed and spleens were collected for the analysis.

After 3 months of experiment statistically significant differences between control and supplemented diet were observed only for SOD. After 6 months results showed significant differences in SOD value, but also Thiobarbituric acid reactive substances (TBARs), GSH-Px and GSH-R. After 12 months all results show significant differences, including Total Antioxidant Status (TAS). The result of the study shows that long term enrichment of the rat diet with four different bioactive substances helps in defense the cells against the effects of oxidative stress.

**Reference**


**SW03.S13–124**

**Opening of mitochondrial megachannel by iron: Competition of iron with calcium more important than oxidative stress**

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The mitochondrial permeability transition (MPT, also “mitochondrial megachannel”) is an interesting phenomenon playing an important role in calcium signaling and cell death. The MPT is triggered by calcium and promoted by oxidative stress. In the body the oxidative stress is often catalyzed by iron or copper. We investigated the induction of the MPT by physiological (micromolar) concentrations of iron. Isolated rat liver mitochondria were initially stabilized with EDTA and bovine serum albumin and energized by succinate or malate/pyruvate. The MPT was induced by 20 µM calcium or ferrous chloride. We measured mitochondrial swelling, the inner membrane potential, NAD(P)H oxidation, iron and calcium in the recording medium. Iron effectively triggered the MPT; this effect differed from non-specific oxidative damage and required some residual EDTA in the recording medium. Evidence in the literature suggested two mechanisms of action for the iron: a) NAD(P)H oxidation due to loading the mitochondrial antioxidant defense systems, and b) uptake of iron to the mitochondrial matrix via a calcium uniporter. Both of these events occurred in our experiments but were only marginally involved in the MPT induced by iron. The primary mechanism observed in our experiments was the displacement of adventitious/endogenous calcium from the residual EDTA by iron. Although artificially created, this interplay between iron and calcium can well reflect conditions in vivo and could be considered as an important and probably fairly general mechanism of iron toxicity in the cells.

**SW03.S13–125**

**The content of metallothioneins and lipid peroxidation in mouse brain: effects of cadmium and nickel ions**

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Cadmium (Cd) and nickel (Ni) are toxic metals that may induce oxidative damage by disturbing the prooxidant-antioxidant balance in the different tissues. Due to its high rate of blood flow, the brain is one of the most sensitive organs to the effect of metals. The present investigation was undertaken to evaluate effects of Cd and Ni ions on the content of metallothioneins (MTs) and on lipid peroxidation in mouse brain.

Experiments were done on 4–6 weeks old outbred mice weighing 20–25 g. The content of MTs was evaluated by the colorimetric method with Ellman’s reagent. Lipid peroxides were measured as malondialdehyde (MDA) formed after reaction with thiobarbituric acid.

It is suggested that MTs may provide protection against metal toxicity, be involved in regulation of concentration of essential metal ions, as well as provide protection against oxidative stress. In this relation our experiments aimed to investigate effects of Cd and Ni ions on the content of MTs in mouse brain after 24 h and 14 days of injections of CdCl₂ or NiCl₂ solution. The data obtained showed that 24 h after exposition of Cd or Ni MTs concentrations in the mouse brain increased by 18% and 25%, respectively. On the other hand, MTs content in mice brain after 14 days of injections of CdCl₂ or NiCl₂ solution was at the control level.

The most reliable method for detecting of oxidative stress is determining of MDA level. Our results indicated that 24 h after injection of CdCl₂ or NiCl₂ solution, MDA content in mice brain was relatively increased by 59% and 54% as compared to control. After 14 days of mice treatment with metals, an increase in MDA content was observed in mouse brain (by 16%) only after injection of CdCl₂ solution. It is interestingly that an increase in MDA content was also observed in mouse red blood cells after 24 h and 14 days of injection of NiCl₂ solution by 40% and 23%, respectively.
Altogether our results indicate that either Cd or Ni induce oxidative stress in brain and confirm the hypothesis that the importance of MT's complex with metals is to protect cells from their toxicity.

**SW03.S14** ‘Mitochondriology’: New Approaches in Bioenergetics (III-S14)

**SW03.S14–1**

SkQ, the first approved mitochondria-targeted medicine: clinical trials and therapy of the dry eye syndrome, an incurable age-related disease

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The process of ageing affects all systems and organs of the body, including eyes. One of the most widely spread eye disease is dry-eye syndrome caused by age-dependent decline of tear quality and quantity due to lachrymal gland degeneration. Thus development of dry-eye syndrome can be regarded as a trait of general ageing of the organism. This assumption is supported by recent observation that such a geroprotector as caloric restriction (CR) slows down the development of dry-eye syndrome [1]. SkQ is an efficient mitochondria-targeted antioxidant comprised of plastoquinone residue linked to triphenylphosphonium cation via decane linker [2]. In numerous studies, this compound displayed activity similar to that of CR [2,3]. Here we summarize results of pre-clinical and clinical studies of both preventive and therapeutic effects of SkQ1 on dry-eye syndrome. In two animal models of the disease and one clinical trial SkQ1-based medicine displayed statistically significant favorable effects that allowed the approval of SkQ1-based eye-drops Vizomitin as an efficient ceratoprotective medicine in Russia. Further clinical studies of Vizomitin now continue in Russia, Ukraine and US.

**References**


**SW03.S14–2**

Mitochondrial dysfunction in human disease

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There is strong evidence implicating mitochondrial dysfunction in the pathogenesis of neurodegenerative diseases. In Friedreich’s ataxia the genetic defect occurs in a mitochondrial protein. In the case of Alzheimer’s disease (AD), Parkinson’s disease (PD), and Huntington’s disease (HD) there is transcriptional dysregulation of PGC1-alpha, a master regulator of mitochondrial biogenesis and expression of antioxidant enzymes. In PD a deficiency of parkin leads to a buildup of PARIS, which inhibits PGC1-alpha expression, and alpha-synuclein also impairs the expression of PGC1-alpha. The autosomal recessive genes, parkin and PINK1, play key roles in mitophagy, the pathway for removal of damaged mitochondria. DJ1 modulates oxidative damage within mitochondria. In HD, impairment of PGC1-alpha leads to a reduction in numbers of mitochondria in basal ganglia spiny neurons, which correlates with reduced PGC1-alpha. Impairment of mitochondrial trafficking and turnover also play a role in neurodegenerative diseases. Mutant Huntington binds to DRP1 and increases its GTPase activity, and similar effects have been reported in AD. Mutant SOD1, a cause of autosomal dominant ALS, forms aggregates which bind to the outer mitochondrial membrane, impairing the activity of the VDAC channel, as well as impairing protein uptake. Transgenic mouse models of neurodegenerative diseases have been valuable for testing and developing new therapies. Creatine, a precursor of phosphocreatine, is neuroprotective against MPTP and in transgenic mouse models of HD and ALS. It is currently being tested in phase three clinical trials. Co-enzyme Q is an important component of the electron transport chain, and an antioxidant. A phase three clinical trial in PD was unsuccessful, however, trials in HD and Friedreich’s ataxia are continuing. Selective mitochondrial-targeted antioxidants include the peptides SS31 and SS20, and XJB-5–131, which are neuroprotective in transgenic mouse models of ALS and Huntington’s disease. Activation of the nrf2ARE transcriptional pathway is neuroprotective against MPTP, and in transgenic mouse models of AD, HD, and ALS. Dimethylfumarate activates the nrf2ARE pathway, and is protective in multiple sclerosis, and in transgenic mouse models of HD. PPAR agonists which activate PGC1-alpha, a transcriptional regulator of mitochondrial biogenesis and expression of antioxidant enzymes, protect in transgenic mouse models of AD, HD and ALS. These therapeutic approaches targeting mitochondria show great promise for the treatment of patients with neurodegenerative illnesses.

**SW03.S14–3**

Acute phenoptosis: sudden death after a crisis, mediated by mitochondrial reactive oxygen species (ROS)


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Mitochondria are multifunctional structures involved in a number of energy-producing and transducing processes and beyond these, Participation of mitochondria in cell death has received significant support while recent data point to their role in programmed organ failure and organismal death. The clearly programmed syndrome of multiple, simultaneous failure of a number of organs (MOF) is obviously a particular example and a source of phenoptotic death of a system. Histological examination often shows no signs of necrotic or apoptotic cell death in a failed tissue, however, signs of mitochondrial dysfunction have been repeatedly described. The mechanisms of mitochondria-mediated phenoptosis may include generation of ROS and damage-associated molecular patterns (DAMPs) by mitochondria. DAMPs have been shown to mediate a fatal systemic inflamma-
In the intermediate stage between the initial damage and MOF. However, in the syndrome of systemic inflammatory response observed in trauma or hemorrhagic shock the blood is sterile. The clinical picture of this phenomenon is not distinguishable from that of bacterial sepsis and mortality is very high in both cases. A unique role in a deadly cascade resulting in organism failure belongs to the kidney. It has been known that any pathology accompanied by acute renal failure increases mortality by 15–60%. In our experiments with rats when one of the two kidneys is removed and the remaining kidney is exposed to ischemia/reperfusion, acute renal failure develops. However, in rats pre-treated with the mitochondria-targeted antioxidant SkQ1 the severity of renal failure is significantly reduced. In addition, the other mitochondrial antioxidant, SkQ1, did not improve renal function although both antioxidants almost completely abolished animal death which was as high as 70% in the absence of the antioxidants. We conclude that contrary to popular belief it was not renal failure that was the cause of animal death but some control system which is the target of used antioxidants. We speculate that it is the immune system involved in mitochondrial signaling that may be responsible for organisal death.

**SW03.S14–4**

**OXYS rats: role of mitochondria in the accelerated senescence**

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The free radical theory proposes that aging is caused by damage to macromolecules by mitochondrial reactive oxygen species (ROS). However, recent findings suggest that ROS generation is not the primary or initial cause of aging. Clearly, that mitochondrial function regulates the rate of aging, but the underlying mechanisms remain unclear. We showed that the useful tool to study of ageing is accelerated senescence OXYS rats that spontaneously developed pathological phenotypes similar to human geriatric disorders including cataract, AMD-like retinopathy, sarcopenia and cerebral dysfunctions related with accumulation of β-amyloid (βA). Most of this signs develop to the age of 3 month before enhanced accumulation of molecular damage in tissues. Progressive mitochondrial dysfunction is considered as a possible cause of accelerated senescence in OXYS rats after detection of the functional disturbances in the liver mitochondria (Shabalina et al., 1995). Here by electron microscopy we show increasing with age ultrastructural alterations of mitochondria in the OXYS rat’s tissues include decrease in volume and surface densities and appearance of mitochondria with destructed cristae and lysed matrix correspond to the de-energized state, which is specific for mitochondria incapable of transforming the energy of oxidative reactions. In OXYS rats it was first detected that mitochondria-targeted antioxidants SkQ1 (plastoquinonyl-decyltriphenylphosphonium) at nanomolar concentrations is capable not only to prevent age-associated alterations of the hormone levels and immune system, the development of cataract and retinopathy but also can significantly reversed already developed pathological changes in the lens and retina of OXYS rats as well as age-related behavior alterations. In addition, SkQ1 has beneficial effects to learning ability and memory in OXYS rats, prevents accumulation of βA, mitochondrial abnormalities and reduces the level of mtDNA deletions which significantly increased in the hippocampus of OXYS. Effects of SkQ1 not only directly associated with reduction of oxidative damage but with inhibition of mTOR signaling pathway, and thus acts at least in part by the same mechanism as the caloric restriction.

**SW03.S14–5**

**Mitochondria, from molecular mechanisms to pathophyiology**

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The mammalian oxidative phosphorylation system, in mitochondrial cristae, is made up of four (I–IV) redox complexes and an ATP synthase (complex V), with a total number of 85 constituent proteins and a mass of ~2 million daltons. The respiratory chain converts the energy made available by electron flow from NADH to oxygen, in a proton current, which drives ATP formation from ADP and Pi. X-ray crystallographic analysis of the protein structure of the OXPHOS system provides a definite basis for understanding the molecular mechanism of energy transfer. Allosteric coupling in the protein constituents, is the essential attribute which confers to OXPHOS complexes the capacity of interconversion of electrochemical, protonic, mechanical and osmotic forms of energy.

X-ray crystallographic analysis of Savanov et al. provides the structural basis for an allosteric/mechanical mechanism of proton pumping in complex I. Data from our laboratory show a role of allosteric H⁺/e⁻ coupling at the low spin heme a in the proton pump of cytochrome c oxidase. Detailed atomic mechanisms of proton pump in the oxidase are available. The Q-cycle mechanism is a generally accepted model of proton pumping in complex III. There is, however, evidence for redox-linked allosteric involvement of b cytochromes and the Fe-S protein in the proton-motive activity.

In human cells ATP production has to be adapted to current requirements with changes in production levels in tissues. This is achieved by regulatory processes, involving cellular signal networks, in particular the cAMP/PKA primed, PGC-1α transcription cascade. This makes the OXPHOS system vulnerable to pathological factors acting at epigenetic, genetic and protein/cellular levels. OXPHOS deficiency is, in fact, primarily or secondarily, involved in different diseases.

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**References**


**SW03.S14–6**

**Signal transduction and complex I regulation: pathophysiological implications**

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The mammalian complex I is the largest enzyme of the mitochondrial respiratory chain. It is constituted by 45 subunits of which 7 encoded by the mitochondrial genome, the others by nuclear genes. The concerted expression of nuclear and mitochondrial genes is required to maintain complex I at optimal function and prevent the development of the complex I deficiency. The complex I genetic diseases show the relevance of the complex I in human health and disease and its potential as drug target. The complex I dysfunction is involved in several human disorders including neurodegenerative, mitochondrial myopathies and cardiomyopathies. In this last case the hypothesis of an iatrogenic complex I deficiency due to the use of isocoric agents is supported by the data of single case of complex I deficiency in a patient with acute myocardial injury, who had been treated with isocoric agents.

In this study, we report the identification of a new mutation in the complex I subunit A11 of the patient and we discuss the results of functional assays performed on patient's fibroblasts and on a cell line stably transfected with cDNA encoding the novel A11 mutant. The results show that the A11 mutant is not fully functional in vitro.

**Acknowledgments**

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DNA, the synthesis and import into mitochondria of nuclear encoded subunits and their post-translational modifications are essential for the correct function of complex I. Results on the cAMP/PKA-dependent regulation of these processes are presented. We show that cAMP response element-binding protein (CREB), a transcription factor, is present in the mitochondria where it promotes the expression of mitochondrial encoded proteins (1). In mammalian cells there are subcellular pools of cAMP and PKA (2,3). In response to extracellular effectors, the plasma membrane adenyl cyclase (AC) produces cAMP in the cytosol. A bicarbonate-activable soluble AC (sAC) produces cAMP in the mitochondrial matrix. In fibroblast cell cultures, PKA-mediated phosphorylation of the NDUF5 subunit of complex I rescues the activity of the oxidatively damaged complex (4). In vitro mitochondrial import experiments show that PKA stimulates complex I activity by promoting mitochondrial import of the NDUF5 subunit and its assembly in the complex in exchange with the ‘aged’ carboxylated subunit (4). Using KH7, a specific inhibitor of sAC, we show that reduced level of intramitochondrial cAMP causes decrease of complex I activity and inhibits the assembly in the complex of the new imported NDUF5 protein, both effects being reversed by a permeant analogue of cAMP. In conclusion the results show that the cytosolic and mitochondrial cAMP pools cooperate in the regulation of complex I activity by modulating the import/dynamic assembly of peripheral subunits(s). Altered cAMP/PKA system is associated with complex I defects in several neurological disorders, including Parkinson and Down syndrome (5). The elucidation of the role of the cAMP/PKA system in regulating complex I can provide new perspectives for devising therapeutical strategies for diseases associated with complex I defects.

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SW03.S14–7
ATP synthase oligomers and respiratory supercomplexes: structures, functions and superactivities

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Overwhelming evidence indicates the existence of specific respiratory supercomplexes of the proton-pumping complexes I, III and IV as well as of ATP synthase dimers/oligomers in mitochondria of all eukaryotes. The ATP synthase and the other oxidative phosphorylation (OxPhos) complexes do form supramolecular assemblies that have an impact on cell metabolism, mitochondrial morphology, disease, ageing and vice versa. Numerous data support the notion that protein complexes and supercomplexes involved in oxidative phosphorylation (OxPhos) play a crucial role not only in energy metabolism but also in lifespan control. Especially the generation of ATP by the ATP synthase, energized by the respiratory chain complexes, is an important factor for the ageing process as well as for age-related diseases such as Alzheimer’s and Parkinson’s.

Since enzyme activities of OxPhos-complexes assembled as supercomplexes are 2 to >15 times higher than those of the respective individual complexes, the amount, the proportion of the individual vs. specific supercomplexes and the activity of OxPhos (super)complexes will determine the overall performance of the respiratory chain of ATP generation and have to be considered for a systems biological description of energy transformation as well as for elucidating the molecular basis of ageing and the age-associated diseases Alzheimer’s and Parkinson’s Dementia.

ATP synthase superassemblies exist both in animals and plants. In one and the same plant cell, two significantly distinct H+−ATP synthases are housed in separate organelles, mitochondrial and chloroplast ATP synthases. In the green algae C. reinhardtii, for example, an extraordinarily stable arrangement of exclusively dimeric ATP synthases was found in mitochondria, but also chloroplast ATP synthase dimers. A distinct dimerisation mechanism for mitochondrial and chloroplast ATP synthase is suggested. In the organelar context, ATP synthase dimers and oligomers are involved in the formation of mitochondrial cristae. As a consequence, changes in the amount of such superassemblies, as observed during ageing in fungi, worms, rats and human cells, affect mitochondrial structure and function. Alterations in the cellular power plant have a strong impact on energy metabolism and ultimately play therefore a significant role in pathophysiology. Similar to mammals, a correlation between metabolic changes and the amount of the chloroplast ATP synthase dimers exists. The most important question in context with dimerisation of ATP synthases is still rather ambiguous: are there any differences in the activity between monomeric and dimeric/oligomeric species of the ATP synthase? Whereas in the case of the respiratory chain complexes I, III, IV, the assembly to supercomplexes has been proven to boost the catalytic activity, for the ATP synthase dimer this is unknown.

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References

SW03.S14–8
Human diseases with impaired mitochondrial translation

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Mitochondrial respiratory chain deficiencies represent one of the major causes of metabolic disorders that are related to genetic defects in mitochondrial or nuclear DNA. The mitochondrial translation allows the synthesis of the 13 respiratory chain subunits encoded by mtDNA. Altogether, about 100 different proteins are involved in the translation of the 13 proteins encoded by the mitochondrial genome emphasizing the considerable investment required to maintain mitochondrial genetic system. Translation deficiency can be caused by mutations in any component of the translation apparatus including tRNA, rRNA and proteins. Mutations in mitochondrial tRNA and tRNAs have been first identified in various forms of mitochondrial disorders. Moreover
abnormal translation due to mutation in nuclear genes encoding tRNA modifying enzymes, ribosomal proteins, aminoaeryl-tRNA synthetases, elongation and termination factors and translational activators have been successively described. These deficiencies are characterized by a huge clinical and genetic heterogeneity hampering to establish genotype-phenotype correlations hampering an easy diagnosis. One can hypothesized that new techniques for gene identification, such as exome sequencing will rapidly allow to expand the list of genes involved in abnormal mitochondrial translation.

SW03.S14–9
Penetrating cation C12TPP as a possible tool to treat obesity
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Dodecyltriphenyolphosphonium (C12TPP) is a novel cationic compound suggested to facilitate recycling of fatty acids in the mitochondria. This results in transmembrane H⁺ conductance pathways leading to increased proton leakage (Severin et al., 2010).

We studied the effects of C12TPP on brown adipose tissue. Respiration of isolated brown fat mitochondria was initiated by addition of pyruvate + malate or by palmitoyl CoA + carnitine. Uncoupling protein 1 (UCP1)-dependent uncoupling was inhibited by subsequent addition of excess GDP. The addition of C12TPP to this system resulted in uncoupling, which was more prominent in the presence of fatty acids. We also studied the C12TPP effect in brown adipocytes and found that cells pre-treated with C12TPP during 1 h exhibited an increased level of oligomycin-insensitive oxygen consumption. The effects on mitochondria and cells were observed in both wildtype and UCP1-KO mice, and were found to be UCP1-independent.

We also examined in vivo effect of C12TPP in mice (dose 50 μmol/kg/day in drinking water) and observed a significant reduction of body weight as well as body fat content. The food intake was decreased during the first 3 days of treatment and then spontaneously recovered. C12TPP in drinking water did not affect the drinking preference, morphology of the alimentary tract and fat content in feces. To elucidate the effect of C12TPP independent of food intake, all measurements were performed also in a pair-fed group (mice receiving the same reduced amount of food as treated mice ate). We observed that only 30% of the body weight lost by C12TPP-treated mice was due to reduced food intake. C12TPP-treated mice exhibited a reduced respiration quotient, consistently with loosing more body fat than pair-fed mice. In contrast to expectation, C12TPP did not increase the resting metabolic rate as does classical uncoupling agent, dinitrophenol.

Thus, the novel cationic compound C12TPP facilitated uncoupling in brown fat mitochondria and cells and enhanced fatty acid utilization in mice.

Reference

SW03.S14–10
Proton pumping mechanism of bovine heart cytochrome c oxidase
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Cytochrome c oxidase (CcO) pumps protons electrostatically through a hydrogen bond network using positive charges created upon O₂-reduction. Complete loading of four proton-pumping equivalents through a water-channel and into the network in each catalytic turnover is followed by O₂-binding which triggers channel closure to block spontaneous backward leakage of protons during the pumping process. Timely channel closure is critical for determination of the pumping direction. Conformational changes after CO-photolysis, monitored by a newly developed highly sensitive time-resolved infrared system, indicate that migration of CO, within the O₂ reduction site, from Fea3 to CuB induces an intermediate open state of the water-channel by elimination of a bulge conformation at Ser382. These results suggest that CuB facilitates effective pumping-proton collection and timely closure of the channel using a relay system between CuB and Ser382 which senses complete loading of protons into the network.

The present X-ray structural improvements of the oxidized/reduced CcO from 1.8/1.9 Å resolution to 1.5/1.6 Å resolution for searching the structural bases for the pumping-proton collection and storage reveal a large cluster composed of 21 water molecules and a Mg²⁺ site including Glu198. The cluster is tightly sealed sterically against proton exchanges with the outside of the cluster except for a short hydrogen bond network connecting the cluster with the hydrogen bond network through which protons are actively transported (pumped) as described above. Five proton-acceptable groups hydrogen-bonded with the cluster suggest sufficient storage capacity for four proton equivalents. The redox-coupled X-ray structural changes in the electron transfer pathway from CuA, the initial electron acceptor from cytochrome c, to heme suggests that redox-driven effective proton donations from the cluster to the hydrogen bond network for proton pumping are facilitated by Glu198 which bridges CuA and Mg²⁺. These results indicate that the cluster is a crucial element of the proton-pumping system of bovine CcO.

SW03.S14–11
Induction of non-selective permeability of the inner membrane of rat liver mitochondria by ω-dioic acids
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We studied the ability of ω-hedecanedioic acid (HDA), which is formed in the liver by the hydrogenation of palmitic acid [1], to induce cyclosporin A (CsA)-insensitive non-specific permeability of the inner membrane of rat liver mitochondria.

It was established that the addition of 10–30 μM HDA to energized (succinate plus rotenone) rat liver mitochondria loaded with Ca²⁺ or Sr²⁺ leads to a high-amplitude swelling of organelles and release of Ca²⁺(Sr²⁺) from the matrix which is an evidence of the pore opening in the inner membrane of mitochondria. We have shown that the opening of a pore causes release of cytochrome c from organelles. CsA didn’t inhibit the observed processes. The calcium uniporter inhibitor ruthenium red, Ca²⁺(Sr²⁺) chelator EGTA, nucleotides (ATP, ADP) pre-
vented the pore opening. Unsaturated fatty acids (oleic, linoleic) and spermine also have inhibitory effects. Bovine serum albumin (BSA) inhibited HDA-induced swelling. However, at the same BSA: Fatty acid molar ratio inhibitory effect of BSA is much less pronounced when we used HDA as the pore inducer than palmitic acid. Apparently this is due to the fact that BSA binds ω,ω'-dioic acids weaker than their monocarboxylic analogues [2]. In the experiments with 36 kDa polyethylene glycol we found that the pore induced by Ca²⁺ and HDA can close spontaneously. In light of these results, the accumulation of ω,ω'-dioic acids in the liver cells which occurs in a number of pathologies [3] can be considered as one of the factors leading to their death.

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References

SW03.S14–12 Mitochondrial targeting of RNA: alternative mechanisms of translocation
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Mitochondria import from the cytosol several types of small nuclear DNA encoded RNAs, which participate in mtDNA replication, RNA processing or translation. Being the unique natural way of nucleic acids delivery into mitochondria, this pathway represents an obvious interest as a tool to address mitochondrial diseases, especially a vast group of incurable pathologies associated with mutations in mtDNA. Nowadays, the mechanisms of RNA mitochondrial import are not understood in details and appear to have significant variations among species. We addressed, in a comparative way, two evolutionary distant species: yeast S. cerevisiae and humans, to identify membrane associated proteins participating in RNA translocation in the organelle. We demonstrated, by biochemical and genetic approaches, that RNA molecules can use at least two alternative mechanisms to cross the outer mitochondrial membrane – either involving the pre-protein import machinery (TOM complex) or porines (VDAC1 or VDAC2, Voltage Dependent Anion Channel). Furthermore, the important role of the mitochondrial inner membrane associated protein PNPT1 (polynucleotide phosphorylase, PNPase) as a part of RNA translocation machinery was confirmed by studying biochemical and molecular effects of a pathogenic PNPT1 mutation.

This study was supported by ANR, FRM, AFM, Labex MitoCross and LIA ARNmitocure.

SW03.S14–13 Mitochondria in cell differentiation and programmed cell death: mitochondria-targeted antioxidant SkQ1 stimulates wound healing

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Mitochondria-targeted antioxidants are the powerful tools for studies on the role of mitochondrial reactive oxygen species (mtROS) in physiological processes. We have shown that mitochondria-targeted antioxidant SkQ1, (10-(6'-plastoquinonyl) decyltriphenylphosphonium) stimulated healing of full-thickness dermal wounds in old (28 months) mice and in db/db mice with type II diabetes. Prolonged treatment with SkQ1 (250 nmol/kg per day) accelerated inflammatory as well as regenerative phases of wound healing. SkQ1 stimulated transition from neutrophil infiltration to accumulation of macrophages, formation of granulation tissue containing myofibroblasts, vascularization, and epithelization of the wound. In endothelial cell culture SKQ1 prevented disorganization of cytoskeleton and intercellular contacts as well as surface expression of the neutrophil adhesion molecule ICAM1 induced by hyperglycemia and pro-inflammatory cytokine TNFa. SkQ1 also inhibited apoptosis induced by high doses of TNFa. These effects indicated the key role of mtROS in inflammatory response of endothelium and could underlay the anti-inflammatory effect of SkQ1. In the in vitro wound model SkQ1 stimulated movement of subcutaneous human fibroblasts into the ‘wound’ and myofibroblast differentiation. The stimulation of movement depended on modulation of Rho/Rac signaling, while the differentiation was mediated by activation of TGFβ.

Anti-inflammatory, vasculoprotective and pro-differentiation effects of mitochondria-targeted antioxidants make them promising candidates for regenerative medicine.

SW03.S14–14 Multistationary and oscillatory modes of free radicals generation by the mitochondrial respiratory chain
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The mitochondrial electron transport chain transforms energy satisfying cellular demand and generates reactive oxygen species (ROS) that act as metabolic signals or destructive factors. Therefore, knowledge of the possible modes and bifurcations of electron transport that affect ROS signaling provides insight into the interrelationship of mitochondrial respiration with cellular metabolism. A bifurcation analysis of a sequence of the electron transport chain models of increasing complexity was used to analyze the contribution of individual components to the modes of respiratory chain behavior. Our algorithm constructed models as large systems of ordinary differential equations describing the time evolution of the distribution of redox states of the respiratory complexes. The most complete model of the respiratory chain and linked metabolic reactions was validated by a specific experiment: the model predicted and experiment confirmed that mitochondria produce more ROS at low succinate concentration and less ROS at high succinate levels than swelled mitochondria. A numerical bifurcation analysis revealed qualitatively different types of multistationary behavior and sustained oscillations in...
SW03.S14–15
Effect of lipophilic cations on yeast mitochondria
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Mitochondria are believed to be the general source of reactive oxygen species in the cell. Oxidative stress caused by mitochondrial dysfunction leads to accelerated aging, the development of pathologies, including diabetes, cardiovascular disorders, heart attack, stroke, glaucoma, cataracts, osteoporosis, neurodegenerative and other age-related diseases. Numerous attempts have been made to slow down these diseases by antioxidants. Mitochondria-targeted lipophilic antioxidants offer advantages over conventional water-soluble antioxidants as they are transported into and accumulated within cells and mitochondria in conformity with the membrane potential generated on the cytoplasmic or mitochondrial membrane, respectively. As a result, their concentrations in mitochondria would increase several orders of magnitude in comparison with the initial low, nontoxic nanomolar and sub-nanomolar concentrations. Tightly-coupled yeast mitochondria are an adequate model for elucidating the mechanism of their action. Moreover, they have some advantages over animal mitochondria as they are practically devoid of endogenous respiration, which permits investigation of the oxidation of individual substrates and, as we showed (Trendeleva et al., 2011a, b), lack a Ca\(^{2+}\)/dependent pore, which facilitates interpretation of the data obtained. We have studied a number of mitochondria-targeted lipophilic antioxidants including 10-(6'-plastoquinonyl)deceylriphenylphosphonium (SkQ1), 10-(6-plastoquinonyl)deceylrhodamine-19 (SkQR1), 13-[9-(6-plastoquinonyl) nonyloxybenzyl]berberine (SkQBerb), 13-[9-(6-plastoquinonyl) nonyloxybenzyl]palmatine (SkQPalm) and their analogs lacking plastoquinol moiety. It was shown that these conjugates (SkQBerb, SkQPalm and their analogs lacking plastoquinol moiety, C10Berb and C10Palm) penetrated across planar bilayer phospholipid membrane in their cationic forms while their anionic forms did not penetrate. Reduced forms of SkQBerb and SkQPalm inhibited lipid peroxidation in isolated mitochondria at nanomolar concentrations. In isolated mitochondria and in living cells, berberine and palmatine moieties were not reduced, so antioxidant activity belonged exclusively to plastoquinol moiety. In human fibroblasts SkQBerb and SkQPalm prevented fragmentation of mitochondria and apoptosis induced by hydrogen peroxide.

The aliphatic conjugates of berberine and palmatine (as well as the conjugates of triphenylphosphonium) induced proton transport mediated by free fatty acids (FA) both in the model and mitochondrial membrane. In mitochondria this process was facilitated by the adenine nucleotide carrier.

SW03.S14–16
The novel mitochondria-targeted antioxidants – derivatives of plant alkaloids berberine and palmatine
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The novel mitochondria-targeted compounds composed entirely of natural constituents were used for a design of mitochondria-targeted compounds that were tested in model lipid membranes, isolated mitochondria and in culture of living human cells. Berberine and palmatine, penetrating cations of plant origin were conjugated by nonyloxybenzylmethyl residue with plant electron carrier and antioxidant plastoquinone. It was shown that these conjugates (SkQBerb, SkQPalm and their analogs lacking plastoquinol moiety, C10Berb and C10Palm) penetrated across planar bilayer phospholipid membrane in their cationic forms and accumulated in isolated mitochondria or in mitochondria of living cells in culture due to membrane potential negative inside. Reduced forms of SkQBerb and SkQPalm inhibited lipid peroxidation in isolated mitochondria at nanomolar concentrations. In isolated mitochondria and in living cells, berberine and palmatine moieties were not reduced, so antioxidant activity belonged exclusively to plastoquinol moiety. In human fibroblasts SkQBerb and SkQPalm prevented fragmentation of mitochondria and apoptosis induced by hydrogen peroxide.

The aliphatic conjugates of berberine and palmatine (as well as the conjugates of triphenylphosphonium) induced proton transport mediated by free fatty acids (FA) both in the model and mitochondrial membrane. In mitochondria this process was facilitated by the adenine nucleotide carrier.
full picture of the domains of the protein that are important for potential KHE activity in human LetM1.

**SW03.S14–18**

**Substrate induced transcriptional upregulation of mitochondrial proteases: the mitochondria response to matrix accumulation of steroidogenic acute regulatory (StAR) protein**

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Steroidogenic cells express a mitochondrial matrix protein known as steroidogenic acute regulatory protein (StAR) that is essential for high output production of vital steroid hormones in the adrenal cortex and the gonads. StAR activity facilitates mobilization of cholesterol from the outer to the inner mitochondrial membranes where the sterol serves as substrate for steroid biosynthesis. It is currently accepted that import of StAR to the mitochondrial matrix rather terminates the cholesterol mobilization activity of the protein. Consequently, steroidogenic mitochondria rapidly accumulate exceedingly high matrix content of StAR protein calling for a rapid clearance. Previous and the present studies show that LON protease is the first of several predicted mitochondrial proteases that degrade StAR, including the m-AAA membrane metalloproteinase complex of AFG3L2/SPG7. We show that StAR accumulation in the mitochondria generates a yet to be found mitochondria-to-nucleus signaling leading to activation of the protease genes engaged in StAR degradation. Such adaptive changes of the mitochondrial protease content is not only physiologically relevant in steroidogenic ovary cells in vivo, but also constitute a functional response of the organelles in any cell type made to overexpress StAR. StAR induced transcriptional response does not include protease genes that are not involved in StAR degradation, such as CLP protease that resides in the matrix similarly to LON. Consistent with such specificity hallmark is the fact that in order to upregulate LON, AFG3L2 and SPG7, StAR must be present in the matrix compartment; StAR mutants that do not enter the matrix, such as C28-StAR or N47-StAR are ineffective. Also, the transcriptional response to StAR is not dependent on the cholesterol mobilization activity of StAR since a naturally occurring loss-of-function StAR mutant, A218V, induces the protease gene activation equally well as the wild-type protein. Taken together, this study unraveled a novel regulatory loop, whereby acute accumulation of an apparently nuisance protein in the matrix provokes mitochondria-to-nucleus signaling that, in turn, activates specific transcription of genes encoding the mitochondrial proteases relevant for the enhanced clearance of the protein client.

**SW03.S14–19**

**The effect of ionic strength of incubation medium on the cytochrome c release from liver mitochondria under conditions of the pore opening by γ,ω-hexadecanedioic acid**

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Recently we found that the γ,ω-hexadecanedioic acid (HDA) induces the opening of a cyclosporin A (CsA)-insensitive pore in the liver mitochondria loaded with Ca$^{2+}$ or Sr$^{2+}$. It is interesting to find out how release of cytochrome c from the liver mitochondria induced by the opening of a HDA-induced pore depends on the ionic composition of incubation medium.

We used two different incubation medium: (i) 125 mM KCl (KCl-medium) and (ii) 20 mM KCl + 200 mM sucrose (sucrose medium). It was found that in the presence of 1 μM CsA sequential addition of 200 mM Ca$^{2+}$ and 20 mM HDA to the liver mitochondria leads to a high-amplitude swelling of organelles, indicating the pore opening in the inner membrane. In this case, the rate of swelling of the liver mitochondria is greater in KCl-medium, and the amplitude of swelling is greater in sucrose medium. The swelling of organelles in sucrose medium is accompanied by almost maximal stimulation of the liver mitochondria respiration. While the swelling of the liver mitochondria in KCl-medium does not change the rate of respiration, which is associated with inhibition of the electron transport along the respiratory chain. The subsequent addition of 10 μM cytochrome c to the liver mitochondria leads to an almost maximal stimulation of the respiration. Measurement of the cytochrome c release showed that during the induction of pore by HDA more protein released in KCl-medium than in sucrose medium. It is assumed that due to the high ionic strength of KCl-medium both cytochrome c which freely diffuses into the intermembrane space is released. In the latter case, a massive release of protein from the liver mitochondria leads to an almost complete blockade of the electron transport along the respiratory chain and consequently to a violation of the energy functions of the liver mitochondria.

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**SW03.S14–20**

**Agmatine and alpha-methylagmatine: permeabilizing the outer mitochondrial membrane**

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Agmatine a molecule belonging to the polyamine family, is a biogenic diamine with about two positive charges at physiological pH, which acts as a neuromodulator and neurotransmitter. It has different biochemical effects among which the induction of ornithine decarboxylase (ODC) and the inhibition of proliferation by suppressing intracellular polyamine levels [1]. Agmatine exhibits opposite effects at the level of the mitochondrial permeability transition (MPT) in isolated mitochondria, that is inhibition at high concentrations or induction at low concentrations [2]. This latter process involves the formation of the transition pore at contact sites between the mitochondrial outer and inner membranes, causing a permeability increase of the membranes [3].

Considering that the MPT induction is strongly correlated with the release of the pro-apoptotic factors, reasonably high agmatine concentrations should not be able to induce their release. Instead, we observed that, while this amine is inhibiting the MPT, it causes the release of some pro-apoptotic factors such as cytochrome C (cyt C) and SMAC/DIABLO, but not Apoptosis Inducing Factor (AIF). This differential release induced by agmatine could be correlated with the localization of the pro-apoptotic factors, hypothesizing that the permeabilization of the only mitochondrial outer membrane (MOMP), but not of the inner membrane, can cause the release of the intermembrane...
space proteins (cyt C and SMAC/DIABLO) and not of transmembrane proteins of the inner membrane (AIF) [4,5]. Moreover we observed that a more stable agmatine analogue, alpha-methylagmatine (α-Meagmatine), is a powerful inhibitor of the MPT, if compared with the amine. Thus, first of all, we investigated if the transport mechanism of the analogue can be mediated by the same agmatine transporter. Then, by considering the pathophysiological significance of the pro-apoptotic factors release, we performed a comparison between the effects of agmatine and the analogue on the above release.

This work was done in collaboration with the Department of Molecular Sciences and Nanosystems, ‘Ca’ Foscari University of Venice.

References

SW03.S14–21
The mechanism of spermine cycling across the inner mitochondrial membrane and its pathophysiological implications
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Spermine penetrates the mitochondrial matrix by an electrophoretic mechanism requiring high membrane potential and exhibiting an apparent exponential force-flux relationship. This process takes place by a channel having two asymmetrical energy barriers and requiring an enthalpy of 86 KJ/mol. This transport does not reach the Nerstian equilibrium as an efflux mechanism is operating causing a spermine cycling across the energized mitochondrial membrane in energized conditions. This efflux cannot utilize the uptake channel as mitochondria cannot develop high positive potentials ‘in vivo’ thus the barriers to overcome are too high for permitting its exit.

Spermine is released when the shift ∆Ψ →∆pH (∆Ψ decreases, ∆pH augments) caused by its electrophoretic transport overcomes a critical threshold. The presence of exogenous phosphate, whose transport increases ∆Ψ, decreases the rate of efflux. Instead the presence of exogenous spermine enhances its efflux by favoring the above shift. The causes inducing spermine efflux are a drop in ∆Ψ that weakens the force retaining the polyamine in the matrix, and ∆pH enhancement (more basic inside, more acidic outside) which deprotonates spermine by favouring its interaction with the acidic pH outside. In conclusion spermine uptake is driven by ∆Ψ while its efflux is driven by ∆pH. These processes establish a continuous cycling of spermine across the membrane that can be modulated by agents able to influence both ∆Ψ and ∆pH. Matrix spermine level imposed by its cycling rate has very important pathophysiological implications. It regulates the activity of pyruvate dehydrogenase and increases the mitochondrial fluxes of Ca2+ and phosphate. This later effect is determinant in the induction and regulation of mitochondrial permeability transition and the apoptotic pathway triggering. The augment of Ca2+ matrix level also increases the activity of the Ca2+–dependent enzymes of Krebs cycle that together the activation of pyruvate dehydrogenase assume a prominent role in energy transduction.

This work was done in collaboration with the Department of Molecular Sciences and Nanosystems, ‘Ca’ Foscari’ University of Venice.

SW03.S14–22
Testicular mitochondrial bioenergetics is altered in pre-diabetes induced by a high-energy dietin rats
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Pre-diabetes is characterized by mild-hyperglycemia and constitutes a high-risk factor for type 2 diabetes. It leads to metabolic changes that may be reflected at testicular level. The knowledge on pre-diabetic state-mediated impairment in overall testicular bioenergetics remains scarce. Hence, we hypothesized that pre-diabetes impair testicular mitochondria function. For this purpose, we induced a pre-diabetic state in rats using a high-energy diet (HED) and studied testicular mitochondrial DNA (mtDNA) integrity and mitochondria function, as well as energy levels. mtDNA content was determined by qPCR and mitochondrial function was evaluated by assessing the mitochondrial respiratory chain complexes activity. Adenine nucleotides, as well as adenosine and its metabolites (inosine and hypoxanthine) concentrations were determined by reverse-phase HPLC. HED fed rats showed increased glycemic levels, impaired glucose tolerance and hypoinsulinemia. Moreover, an imbalance of intratesticular and serum testosterone levels was observed. HED rats showed a significant decrease in testicular mtDNA content, although no differences were observed on mitochondria content and integrity. Regarding mitochondrial respiratory chain, testes from HED animals presented an increased complex I activity, whereas complex III activity was decreased. Adenylate energy charge was also affected, as both ATP and ADP levels were decreased in testis of HED rats. Moreover, AMP levels were significantly increased, lowering the ATP/AMP ratio. Our results show that pre-diabetes is responsible for a decrease in testicular mtDNA integrity and impaired testicular mitochondrial respiratory chain function. Mitochondrial complex III is severely affected in pre-diabetic rats and might be responsible for the decreased testicular adenylate energy charge observed. This is the first report giving new insights into overall testicular bioenergetics at this very earlier stage of potential diabetes development.

SW03.S14–23
Characterization of the secondary mitochondrial dysfunction by multi-scale shifts of plasma analytes
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Metabolic profiling is the most informative laboratory characteristic of organism phenotype state [1]. The secondary mitochondrial dysfunction is characterized by deep changing in substrates utilization from plasma and mitochondrial proteins loosing from organelle.
The aim of the present study was to evaluate metabolic shifts as a characteristic of mitochondrial dysfunction (MD) in patients with activation of intravascular coagulation and fibrinolysis.

The results of testing blood plasma samples from patients (n = 34, from 50 to 83 years) with D-dimer level above 500 ng/ml and lactic acid (LA) concentration above 2.2 mM were compared with those of healthy donors (n = 20, from 55 to 61 years). The concentrations of cytochrome C (CytC, ELISA, eBioscience, Austria), total homocysteine (tHcy) and rates of the amino acids (AA, HPLC) were determined. Statistical analysis of the data included descriptive statistics (data are presented as medians and interquartile range), comparison (Mann–Whitney’s test), correlation (Spearman’s criterion), and factor analysis.

The rates of analytes in a group of patients were: CytC 77(28–285) ng/l, LA 3.0(2.3–4.1) mM, tHcy 13(9–19) μM, Ala 538(431–705), Gly 298(203–365), Ile 104(90–124), Leu 179(145–202), Val 203(178–248) μM. The tHcy level in patient species was significantly higher than in donors’ plasma 11(8–12) μM. Median values of Ala, Ile, and Leu in patients group were greater than the correspondent 75 percentiles rates of the reference group. Besides that, the positive relationship between CytC concentration and the unidirectional shifts of Ile and Gly in the patients group (r = 0.41 and r = 0.42 respectively; p < 0.05) confirmed the presence of MD which was established by LA level. The 30% of patients had Ala/(Phe + Tyr) ratio value >4, which had been considered as an additional sign of MD [2]. This ratio was positively correlated with LA level in patients group (r = 0.47; p < 0.05). Some importance for the progression of intravascular coagulation has elevated tHcy level. Positive correlation of tHcy and CytC (r = 0.49; p = 0.03) demonstrated the association of mitochondrial and endothelial dysfunction. Factor analysis distributed the investigated parameters into four groups (Ft). The Ft1 included the 14 AA. The Ft2 included LA and a ratio of Ala/(Phe + Tyr), and the Ft3 included Arg, Orn, and Cit, to the Ft4 referred tHcy, CytC, Gly, Ile, and (α)Ser. It is known that the conversion of Ser to Gly is a reaction of the one-carbon fragment formation. This reaction partially uses for Hcy remethylation. Coordinated Gly and Ile shifts correspond to their catabolism in mitochondrion.

Thus, the observed MD signs in the patients’ plasma included the AA shifts associated with the mitochondrial decarboxylation pathways and Krebs cycle precursors or intermediates. The study revealed the characteristic plasma metabolite shifts which should be taken into account for MD diagnostic criteria working up. Evaluation of a multi-scale plasma proteomic and metabolomic shifts with modern analytical techniques will be find a further development in clinical biochemistry.

References

Formation of copper metabolic system in adrenal glands during development and link between adrenal glands and copper metabolism in liver

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Copper is a trace element. Being a cofactor, it performs several important functions in mammals. The molecular mechanisms of copper traffic within cells are known in much detail; but its transport to organs is less studied. Liver absorbs, distributes, and excretes copper; therefore, it plays the role of the central organ of copper metabolism. It is not known, how copper transport in the whole body and its metabolism in liver are controlled by other organs.

Our preliminary data on copper and silver [Ag(I) ions are isoelectronic to Cu(I)] distribution in mammalian organs indicated that adrenal glands might have a copper metabolic mechanism that differs from most other tissues. In this study we demonstrate that removal of adrenal glands in rats results in two important changes in copper metabolism in the liver. First, this leads to a decrease in copper excretion from hepatocytes, which is manifested as an elevation of total copper concentration in liver. Second, copper is redistributed within the liver cells. Accumulation of copper in cytosol is not associated with growth of holo-SOD1 content. In contrast, SOD1 activity dramatically decreases. An increase in the copper content in Golgi complex is in accordance with elevated production of secretory copper-containing protein ceruloplasmin. Conversely, copper concentration in mitochondria decreases; this also coincides with changes in mitochondrion structure as evidenced by sedimentation analysis. Furthermore, 2D electrophoresis of hepatocyte mitochondrial fraction reveals changes in mitochondrial proteome of adrenalectomized rats. Besides, we compared expression levels of copper-associated genes in adrenal glands and liver at different stages of ontogenic development in order to characterize properties of adrenal gland specific copper metabolic system and track its interaction with copper homeostasis in liver.

We discuss the involvement of adrenal glands into maintenance of copper homeostasis in a mammalian organism.

Inherited variation in mtDNA in SHR-mtF344 versus SHR conplastic strains is associated with reduced OXPHOS enzyme levels, insulin resistance, left ventricular hypertrophy and cardiac dysfunction

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Common inbred strains of the laboratory rat can be divided into four major mitochondrial DNA (mtDNA) haplotype groups represented by the SHR, BN, LEW and F344 strains. In the current study, we investigated the metabolic and hemodynamic effects of the SHR versus F344 mtDNA by comparing the SHR versus SHR-mtF344 conplastic strains that are genetically identical except for their mitochondrial genomes. Altogether 12 amino acid substitutions in protein coding genes and seven single nucleotide polymorphisms in tRNA genes were detected in F344 mtDNA when compared to SHR mtDNA. Analysis of oxidative phosphorylation system (OXPHOS) in heart left ventricles (LV), muscle and liver revealed reduced activity and content of several respiratory chain complexes in SHR-mtF344 conplastic rats when compared to the SHR strain. Lower function of OXPHOS in LV of conplastic rats was associated with significantly increased LV mass which was independent on blood pressure and reduced fractional shortening. In addition, conplastic rats exhibited reduced sensitivity of skeletal muscles to insulin action and impaired glucose tolerance. These results provide evidence that inherited alterations in mitochondrial genome, in the absence of variation in the nuclear genome and other confounding factors, predispose to insulin resistance and left LV hypertrophy.
SW03.S14–26
Paternal inheritance of mitochondrial DNA and modeling human mitochondrial diseases in animals
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Mutations in mtDNA cause disruption of energy metabolism and occurrence of so called OXPHOS diseases. Knowing regularities of mutant mtDNA inheritance and distribution among organs is needed to clarify pathogenesis of OXPHOS diseases. Few successful animal models of an OXPHOS disease are hard to be reproduced and the result not always resembles human pathology. Looking for a reliable approach to modeling OXPHOS diseases with predictable distribution of mutant mtDNA among tissues we obtained laboratory mice that develop from zygotes injected with human mitochondria. Human and murine mtDNA coexisted and were transmitted by transmitochondrial (TM) mice in at least five generations along the maternal lineage. Human mtDNA caused no alterations of energy metabolism and its distribution among organs could be traced. In mouse embryos human mitochondria fuse with host organelles as judged by confocal microscopy. Nonrandom distribution of foreign mtDNA among organs in TM mice suggests its segregation. Monte Carlo modeling supported this notion and provided the number of segregation units 11–13. The postulate of strict maternal inheritance of mtDNA in mammals so far remains stable. TM mice obtained in our study of maternal inheritance were used to learn if human mtDNA is transmitted by males to their progeny. A few lineages founded by TM males were obtained in some of which human mtDNA was transmitted by males in five successive generations. Distribution of paternally transmitted foreign mtDNA among organs differed noticeably from maternal inheritance, mainly by the frequencies of various organs becoming targets. Mathematical analysis again pointed at segregation of mtDNA. Replication of human mtDNA in murine tissues indicates that the integral mitochondrial machinery was not altered. This is the first observation of mtDNA transmission along the paternal lineage in more than two generations. Persistence of paternal mtDNA in generations of animals suggests that its elimination is not inevitable. Of all injected zygotes 14–17% develops into TM mice, and frequencies of foreign mtDNA occurrence in various organs were calculated. Our data allow correct modeling of OXPHOS diseases bearing a desired pathological phenotype, with paternal inheritance taken into account.

SW03.S14–27
Functional impact of mitochondrial complex I deficiency in fibroblasts of patients with m.3697G>A mutation in MTDN1
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Complex I (CI, NADH: ubiquinone oxidoreductase) is the first and the most complicated enzyme of the mitochondrial oxidative phosphorylation system (OXPHOS). In human it is composed of 45 subunits. Seven of them are coded by mitochondrial DNA and 38 are coded by nuclear DNA. m.3697G>A mutation is responsible for amino acid change in conserved position of ND1 subunit, which causes defects in CI function followed by serious syndromes with poor prognosis.

SW03.S14–28
A mitochondrial biosensor for studies of molecularity of rate-limiting step of pore formation by alamethicin, mastoparan and melittin
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There is increasing interest in medical use of antimicrobial pore formers – melittin, a 26-residue peptide isolated from Apis mellifera bee venom, mastoparans, 14-residue peptides from wasp venom, and alamethicin, a 19-residue peptide produced by the fungus Trichoderma viride. Melittin could be an effective agent for preventing liver fibrosis [1,2], inhibition of atherosclerosis [3], protection against acute hepatic failure [4]. Melittin restores proteasome function in amyotrophic lateral sclerosis therapy [5]. Mastoparan and melittin regulate hormone receptor interactions, and therefore these peptides can be used in practical endocrinology [6]. Alamethicin synergistically increased the efficiency of enrofloxacin in treating respiratory diseases caused by Mycoplasma pulmonis [7]. These studies together focused on the pharmacological effects of low concentrations of the peptides, but the mechanism of pore formation has been studied insufficiently. We studied the initial steps of pore formation by these peptides in rat liver mitochondria preparations (RLM) generating transmembrane potential (∆Ψ). The degree of steady-state activation of RLM respiration in a ‘potassium’ incubation medium in the presence of valinomycin depends linearly on the potassium transmembrane current (PTC).
induced in RLM. Tightly coupled RLM or mitoplasts in this ‘potassium’ incubation medium and a sensitive oximetric cell has been used as a PTC sensor [8]. This is a noninvasive and contactless determination that requires only the search for a simple dependence between the transmembrane current and certain properties of RLM. In the case of mastoparan, the reaction order was 1.83 ± 0.23. The first steady-state phase of activation of RLM respiration by alamethicin was not detected in ‘Tris’ incubation medium; it appeared only after addition of KCl. This indicates that, as in the experiments on bilayer lipid membranes, PTC during activation was induced by the fraction of lowly oligomeric channels impermeable to Tris" and Mg"++, but permeable to K". The order of the reaction limiting such activation was 1.92 ± 0.07. The ratio of degree of steady-state v4 activation at constant ΔΨ in the presence of alamethicin, melittin or mastoparan in monopotassium and monolithium media was accordingly, 1.59 ± 0.04, 1.12 ± 0.03 and 1.18 ± 0.02. The mobility ratio of K" and Li" in solutions with the same ionic strength was 1.53. Therefore, unlike the other two peptides in the presence of alamethicin cation transmembrane current is not limited by the stage of pre-pore formation. We have shown that in the presence of low concentrations of peptides that form pre-pore (mastoparan and melittin) mitochondria retain ΔΨ at the stationary level. But alamethicin and tetraacetylmelittin have dramatically dissipated mitochondrial ΔΨ, so these peptides may be potentially more hepatotoxic. The understanding of mechanism of peptides pore formation may be useful for evaluation of their toxic effects on mitochondria and comparative testing of perspective pharmaceuticals. 

References

SW03.S14–30
Novel mouse model with decreased levels of somatic cytochrome c in selected cell lineages

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Cytochrome c (cyt c) – a product of the somatic cyt c gene – is an essential component of mitochondrial respiratory chain and an important mediator of the inner apoptotic pathway in vertebrates. The complete Cyt deficiency is lethal in mice and conditional tissue-specific Cycs knock-outs are not yet available. We designed and characterized a novel mouse strain that allowed to combine about 10-fold reduction in Cyt expression (Acyt) and substitution of wild-type protein by K72W Cyt mutant. K72W Cyt mutation doesn’t affect the respiratory chain, but might suppress the inner pathway of apoptosis. These mice initially expressed only wild type cyt c, but in presence of Cre recombinase wt Cyt alleles were excised, creating Acyt genotype. We found at both mRNA and protein levels that this rearrangement resulted in 10-fold decrease of expression of total somatic cyt c, and preferential expression of K72W isoform. Obtained Acyt somatic cells do not express the testicular form of cyt c. The mechanism of Cyt expression knock-down is mediated by alternative splicing with an involvement of neo' cassette which splices with first Cyt exon. Using various Cre deleter mice, several murine strains with decreased expression of cyt c in various targeted cell populations were obtained. For example, using ubiquitous CMV-Cre deleter we found that such knock-down genotype leads to embryonic lethality, similarly to Cyt wt knock-out mice. Using CD4+cre and Mly5-Cre deleter mice we created two mouse strains with decreased expression of cyt c in T cells and in macrophages, respectively. Phenotypes of both Acyt T cell and Acyt macrophage strains were analyzed in vivo and in vitro. Our data support the idea that T cells and macrophages could survive, maintain respiration and proliferate with 10-fold lower amount of cyt c per cell. Thus, conditional Acyt mice provide a new powerful tool for studies in the field of cellular metabolism and apoptosis. Using the plethora of transgenic mice with different tissue-specific Cre-recombinase expression, one can design models for respiratory diseases with significantly decreased expression levels of cytochrome c in certain tissues prone to starvation and damage of the respiratory chain (e.g. neural tissue, muscles and retina).
SW03.S14–31
Developmental changes of gene expression of ATP synthase subunits and assembly factors in human fetal liver and muscle tissues
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One of the most critical developmental moments is the adaptation to oxygen-rich environment after birth when the full OXPHOS activation has to proceed. The key enzyme in OXPHOS is F1F0 ATP synthase (complex V) ensuring the production of most of the ATP in mammalian organisms.

The aim of the study was to characterize expression patterns of complex V subunits (ATP6 – mitochondrial gene, ATP5G2 – nuclear gene) and its assembly factors (ATPAF2, TMEM70) in human fetal tissues during gestation.

RNA were isolated from 35 pairs of fetal liver and muscle samples obtained at autopsy after termination of pregnancy for genetic indications unrelated to OXPHOS deficiency between 13th and 24th week of gestation. The quantification of mRNA was realized by qPCR method.

The most marked differences were observed in expression levels of assembly factors ATPAF2 and TMEM70 between liver and muscle tissues during prenatal development. mRNAs levels of both factors are increasing in muscle tissue (TMEM70: r = 0.73, p < 0.01; ATPAF2: r = 0.58, p < 0.01) meanwhile no statistically significant changes were observed in liver tissue (TMEM70: r = −0.10, p > 0.05; ATPAF2: r = 0.05, p > 0.05). ATP6 and ATP5G2 mRNA levels were increasing significantly in both tissues (liver: ATP6, r = 0.45, p < 0.05; ATP5G2, r = 0.68, p < 0.01 and muscle: ATP6, r = 0.54, p < 0.05; ATP5G2, r = 0.58, p < 0.01).

This work offers the interesting view on the developmental changes in mRNA levels of ATP synthase subunits and assembly factors in two different tissues during second trimester of gestation. It was known that transcript level of ATP5G2 gene correlates with the protein content of complex V. Therefore our results may indicate increasing amount of complex V after 20th week of gestation. Assembly factors were transcribed in a tissue specific manner and their expression may reflect the different phase of tissue maturation.

Understanding the developmental changes in mitochondrial biogenesis pathways may have critical significance in the study and treatment of metabolic disorders.

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SW03.S14–32
Sex-specific mtDNA-protein interactions in a system of obligatory biparental mtDNA inheritance and the exceptional role of perinuclear mitochondria
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Background: Many bivalve mollusks have a maternally (F) and a paternally (M) transmitted mtDNA. The F dominates female and male somatic tissues and the female germ line. Only the M

occurs in the male germ line. The phenomenon, known as Doubly Uniparental Inheritance (DUI), older than 200 MY, violates the rule of maternal inheritance in metazoans and is the best known case of obligatory biparental mtDNA inheritance. The M genome is the only known animal mtDNA that is exclusively paternally inherited.

Observations: We did Electrophoretic Mobility Shift Assays (EMSA) using as probes parts of the control region (CR) of the M mtDNA of Mytilus galloprovincialis. The CR has a domain that is highly divergent between the F and M genomes (the VD1) and a domain that is highly conserved (the CD). Previous work suggests that the CD is the site of replication and transcription control elements and that the VD1 might be the site of sequences that determine whether the genome will be maternally or paternally transmitted. Thus, we used parts of the VD1 and the CD as probes. As a source of a potential binding factor we used cytoplasmic and nuclear extracts from female and male gonads. There was no shift of the M or the F VD1 probe with either the nuclear or the cytoplasmic extract from female gonads and no shift with the cytoplasmic extract from male gonads. But there was a clear shift with the nuclear extract of male gonads. When sequences from the CD of F and M genomes were used as probes, the cytoplasmic extract from both the male and female gonad produced a shift, but the nuclear did not. We obtained no sign for a VD1 coded protein.

Conclusions: Factors in the male gonad bind to M and F VD1 causing the preservation of M and elimination of F in the male germ line. In addition to DUI, our system is a powerful tool for studying perinuclear mitochondria properties that may extend beyond the standard functions of cytoplasmic mitochondria.

SW03.S14–33
Opa1 and Mfn1 are key proteins for muscle cell differentiation in C2C12 cells
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Oxidative damage is believed to underlie the development of many pathological states and aging. The agents responsible for this damage are thought to be reactive oxygen species (ROS) and the main source of ROS production within most of the cells is the mitochondria. Optic atrophy 1 (Opa1) and Mitofusin 1 (Mfn1) are two mitochondrial fusion proteins involved in mitochondrial dynamics. Here we show that Opa1 and Mfn1 loss-of-function impairs myogenic differentiation in C2C12 cells. We observe a 90% decrease in the expression of final markers of differentiation as Caveolin 3 or Myosin Heavy Chain (MHC). Furthermore, there is a significant decrease in Myogenin expression due to a decrease in MyoD activity. Moreover, we find increased mitochondrial hydrogen peroxide levels in living Opal and Mfn1 knocked-down C2C12 myoblasts, suggesting hydrogen peroxide as a key signal that blocks myogenic differentiation. In contrast, Mitofusin 2 (Mfn2), another mitochondrial fusion protein, does not show these changes during the differentiation process. On the other hand, Mfn2 but not Opa1 or Mfn1 loss-of-function, affects the expression of genes involved in contraction and calcium economy. Our data indicate that Opa1 and Mfn1 regulate myogenesis and that Opa1 or Mfn1 loss-of-function causes oxidative stress, which may be a key for dysregulation of muscle differentiation. As conclusion, we propose Opa1 and Mfn1 as potential regulators of muscle differentiation ‘in vivo’.
Mitochondria and exposure to Dibenzofuran: is mitochondria permeability transition a trigger for autophagy in the lung?
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Dioxins and furans are very toxic and exposure to these environmental pollutants, such as fuel constituents, is linked to several diseases.

Mitochondrial function is crucial in cellular homeostasis and keeping a proper energy supply for eukaryotic cells is essential in the fulfillment of the tissues energy-demand. The main objectives of this work concerned Dibenzofuran effects on mitochondrial function.

The effects of Dibenzofuran (DBF) exposure include a markedly increase in the lag phase that follows depolarization induced by ADP, indicating an effect in the phosphorylative system. Experiments performed using carboxyatractyloside (CAT) suggested an interaction of DBF with the ANT carrier. DBF exposure also produces an inhibition of mitochondrial permeability transition (MPT) and an increase in calcium retention capacity, which may also be explained by a putative interaction of DBF with ANT.

These pollutants are related with pulmonary diseases development. A549 human lung cells exposed to DBF showed a decrease in cell proliferation and viability. The prevention of MPT, concomitantly with a decrease in ATP content in cells exposed to DBF, relatively to control, indicate an energetic failure that turn away apoptotic processes in lung cells exposed to the pollutant. Moreover, lung cells in culture exposed to DBF show an alteration in the distribution pattern of LC3 protein, suggesting autophagy processes. Furthermore, we could see an increase in Lysotracker staining, indicative of an increase in lysosomal vacuoles content, that seem to co-localize with mitochondria; thus, these results suggest that exposure to DBF induces autophagy in A549 cells.

Clarifying the role of pollutants in some mechanisms of toxicity, such as unbalance of bioenergetics status and mitochondrial function, as well as the triggering of ‘rescue’ mechanisms such as autophagy, may help to explain the progressive and chronic evolution of diseases derived from exposure to environmental pollutants.

The interplay between the inner membrane formation MINOS complex and MIA pathway responsible for protein transport
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Mitochondria are essential organelles in the eukaryotic cell, which play a crucial role in energy metabolism and regulatory processes. The vast majority of the mitochondrial proteins are synthesized in cytosol and therefore must be imported into this organelle. All precursor proteins utilize the translocase of the outer mitochondrial membrane, TOM, as the main gate to enter mitochondria. After passing through the TOM complex the protein precursors that are directed to the mitochondrial intermembrane space use the specialized MIA (Mitochondrial Import and Assembly) pathway [1]. Mia40, the key component of this pathway, facilitates import and biogenesis of precursor proteins in a redox-dependent manner. Mia40 recognizes precursors emerging from the TOM complex and specifically transfers the disulfide bonds, thus enabling the proteins’ transport, folding and assembly. Our recent studies showed that the protein import activity of Mia40 is regulated by Fcj1 (Formation of Cristae Junctions 1) protein, a key component of the protein complex responsible for maintaining the proper morphology of mitochondria – MINOS (Mitochondrial Inner Inner membrane Organizing System) [2]. We investigate the mechanisms underlying the interplay between the process of inner membrane formation by MINOS complex and protein sorting facilitated by MIA.

References

Expression of ascidian alternative NADH dehydrogenase in Drosophila
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Many mitochondrial diseases are associated with defects in complex I of the electron-transport chain. This complex consists of...
45 subunits encoded by separate genes. Mutations in these genes are associated with serious pathologies manifesting mostly as encephalopathy, cardiomyopathy. Some lower organisms like yeasts, plants, and some animals possess an alternative respiratory chain where the function of complex I is replaced by one or more single-subunit, non-proton-motive NADH dehydrogenases. The ascidian Ciona intestinalis is the organism closest to vertebrates among those possessing an alternative NADH dehydrogenase. The goals of the present work were to express the C. intestinalis alternative NADH dehydrogenase (NDX) in a suitable model organism, investigate its properties and test its potential in gene therapy for complex I defects. The study may also shed light on evolution of organisms and mitochondria, cell functions and the role of mitochondria in metabolism in general.

NDX expressed sequence tags from cDNA clones of C. intestinalis were amplified. The obtained amplicons were spliced together in two serial steps by overlap-extension PCR, resulting in the construct representing the entire coding sequence of NDX. The transgene was inserted into the pMT/V5-His B vector to allow conditional expression in Drosophila S2 cells, under the control of the metallothionein promoter. The NDX-coding sequence was also cloned into a Drosophila transgenic vector pUAST attB, under the control of the GAL4-dependent UAS promoter, and used to create transgenic flies with targeted insertions.

NDX was successfully expressed in S2 cells via copper sulphate induction and, based both on Western blots and immunocytochemistry, was found to be localized in mitochondria. NDX expression in flies, driven by ubiquitously acting da-GAL4, was confirmed by RT-PCR. When present in three copies in the fruit fly genome and driven by da-GAL4, NDX conferred a significant, rotenone-insensitive respiration compared with control flies (without driver). However, when present in only one copy, NDX could not complement the lethality of complex I knockdown.

SW03.S14–38

Study of endogenous fluorescence of mitochondria by fluorescence techniques

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The basis and necessary condition for the use of fluorescence phenomena to monitor vital functions of mitochondria is the presence of fluorophores at the outer mitochondrial membrane, which first comes into contact with the exogenous physical and endogenous influences. Endogenous fluorophores (Phe, Tyr, Trp, cofactors like NADH+$^+$) placed on outer mitochondrial membrane after primary irradiation by UV light emit fluorescence. The aim of our work was to study liver mitochondrial functions after ischemia/reperfusion of the small intestine using fluorescence analysis.

The isolated mitochondria from six sample groups that had undergone three different experimental treatments: a control group, a treatment with ischemia followed by reperfusion of the small intestine (IR) and a 1 h ischemia followed by reperfusion after transplantation of the small intestine (TR). The IR treatment was further subdivided into three groups: 1 h (IR1), 24 h (IR24) and 30 days reperfusion (IR720). Concomitantly, the TR treatment was further subdivided: one group underwent a 1 h reperfusion (TR1) and another group a 6 h reperfusion (TR6) following ischemia and transplantation. Immediately after treatment, mitochondria from control and experimental groups were isolated. The autofluorescence of individual samples of mitochondria was analyzed in respiration medium containing the substrate succinate (using excitation emission matrix, synchronous fluorescence fingerprint) on Luminescence Spectrometer LS55, using 1 cm path length quartz cuvettes at ambient temperature. Data processing was managed by the FL Winlab (Perkin-Elmer) software package.

Our results showed reduction of proteins in all experimental groups compared to the control group. Maximum oxygen deficiency was observed in the samples IR1, IR24 by monitoring the increase of fluorescence intensity in reduced nucleotide NADH+$^+$. Oxygenation during reperfusion restores metabolic processes by supplying oxygen to ischaemic tissue. The highest consumption of oxygen was shown by mitochondrial experimental groups of T/R6 and I/R720 in comparison with control group. The result of increasing oxygen consumption by mitochondria and cells is accompanied by damage of cells. Spectroscopic signals can indicate biochemical changes and these can generally predict the morphological changes observed in histology. Our results showed that the metabolism of liver mitochondria improved significantly after transplantation of the bowl in recipient rats. This work can contribute to understanding the syndrome of multiorgan failure after ischaemia/transplantation of the small intestine.Supported by VEGA 1/0999/11 and APVV - 0252-07.

SW03.S14–39

Studies on targeting NADH dehydrogenase ubiquinone Fe–S 8 (NDUFS8) to mitochondria and rescuing mitochondrial complex I deficiency by HIV-transactivator of transcription (TAT)

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Defects in subunits of mitochondrial complex I are associated with severe diseases, including Leber hereditary optic neuropathy and Leigh syndrome. However, to date, conventional treatment for the majority of genetic-based mitochondrial diseases can only be palliative. Therefore, developing a reliable and convenient treatment approach is in an urgent need. The targeted protein fused with the transduction domain (PTD) of HIV-1 transactivator of transcription (TAT) can be brought into cells by crossing plasma membranes while retaining its biological activities. In this study, we applied the protein transduction concept of TAT with the mitochondrial-targeting capability of the specific leader sequence to generate a therapeutic protein delivery system which can specifically carry targeted proteins into mitochondria. NADH dehydrogenase Fe–S protein 8 (NDUFS8), the first complex I subunit linked to Leigh syndrome, was used as an example here to test this system.

Our findings showed that the exogenously produced TAT-NDUFS8 and NDUFS8-TAT could be specifically delivered into mitochondria and processed into the mature form of NDUFS8. In addition, the results indicated that the mechanism of TAT-NDUFS8 entering mitochondria is not through the well-recognized mitochondrial import pathway [i.e. translocase of the outer membrane (TOM)/translocase of the inner membrane (TIM)]. When cells were co-cultured with TAT-NDUFS8, endosomes were found to be retrieved in close proximity to mitochondria, indicating that TAT-NDUFS8 may enter mitochondria via the endosomes-mitochondria juxtaposition. Next, to mimic the rescue of complex I deficiency, a NDUFS8 expression knockdown cell line (shRNA-C3) was used in functional analyses as the therapeutic model. Treating with TAT-NDUFS8 fusion protein could partially restore the assembly of complex I in shRNA-C3 cells, and the respiratory rate of these NDUFS8 knockdown cells was also increased 79% in the oxygen consumption assay. Our findings provide the possible mechanism of TAT-NDUFS8 entering...
mitochondria and demonstrate the potential of this protein delivery system for therapeutic treatment of mitochondrial disorders.

**SW03.S14–40**

**Structural changes required for temperature adaptation are mediated by a mitochondrial \( \text{H}_2\text{O}_2 \) signal in *Drosophila melanogaster***

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Environmental temperature is the strongest modulator of *Drosophila melanogaster*’s lifespan. At low temperatures, fruit flies live longer; whereas longevity is dramatically shortened at high temperatures. Alterations in lifespan are mediated by changes in the metabolic rate. Such changes are complex, specific and regulated by a genetic program. Mitochondria are central regulators of metabolism so they should play a central role in the adaptation to high or low temperature. Here, we studied which physiological changes are produced in wild type flies when environmental temperature is modified. We found that both oxygen consumption and oxidative stress are increased when temperature is increased. Then, we reduced mitochondrial ROS generation through the expression of Ciona intestinalis alternative oxidase (AOX) that is able to compensate respiratory deficiencies by-passing complexes III and IV. AOX does not have any effect on lifespan at moderate temperatures; however it reduces longevity at very high temperatures. AOX expression prevents the necessary modifications in lipid membranes as well as other parameters related with longevity adaptation. In order to determine the nature of the free radical signal we overexpressed both catalase and sod2 in the mitochondria. As AOX expression, ectopic expression of catalase in mitochondria reduces lifespan at high temperatures which indicates that adaptation to temperature is mediated by a \( \text{H}_2\text{O}_2 \) signal.

**SW03.S14–41**

**Effect of butylrhodamine and dodecylrhodamine on animal mitochondria***

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Effects of butylrhodamine (C\(_4\)R1) and dodecylrhodamine (C\(_{12}\)R1) on tightly-coupled rat liver mitochondria were investigated. At low concentrations, they do not shunt the electron flow via complexes I and II of the mitochondrial respiratory chain, reduce the membrane potential and accelerate respiratory rates in the state 4 respiration upon oxidation of NAD- and flavin-dependent substrates (which is indicative of uncoupling), albeit to varying extend, with C\(_{12}\)R1 being the most effective uncoupler. At low non-uncoupling concentrations they potentiate uncoupling capacities of fatty acids. Notably, both compounds display a significantly wider ‘window’ between the uncoupling and inhibitory effects as compared to the classical uncoupler CCCP, which is a prerequisite for their possible medical application. Treatment with mild uncouplers is a perspective strategy for therapy of a wide range of pathologies related to oxidative stress, senescence and obesity. At higher concentrations C\(_4\)R1 and C\(_{12}\)R1 disrupt the mitochondrial membrane, promote opening of the cyclosporine-sensitive Ca\(^{2+}\)-P\(_1\)-dependent pore and inhibit both ATP hydrolysis and synthesis.

**SW03.S14–42**

**Therapeutic action of the mitochondria-targeted antioxidant SkQ1 on retinopathy in OXYS rats linked with improvement of the alpha-crystallins expression***

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The crystallin proteins have been recently demonstrated to be expressed in normal retina. They are dramatically unregulated by age-related macular degeneration (AMD), the leading cause of blindness in the developed world. Using RNA sequencing (RNA-Seq) we showed that development of AMD-like retinopathy in senescence-accelerated OXYS rats associated with significant decreased expression of the crystallins too. We also found that mitochondria-targeted antioxidant SkQ1 not only prevent development of retinopathy but also cause regression already developed disease’s signs in OXYS rats. Here, we evaluated the connection of its effects with the influence on the crystallins expression in the retina. We confirmed that the retinopathy development in OXYS rats accompanied by downregulation genes CryzB (RT-PCR) in retina. The level of mRNA gene CryzB in OXYS was lower than the control Wistar rats (\( p < 0.05 \)) and decreased with age in the both strain also as a level protein of CryzB (Western blot). These results were supported by immunohistochemical analysis of retina. CryzB is secreted by pigment epithelium and protects photoreceptors from oxidative stress. Mitochondria-targeted antioxidant SkQ1 (from 1.5 to 4 months of age, 250 nmol/kg) prevented RPE abnormalities. The drug increased the level of mRNA of gene CryzB and its protein in retina of OXYS rats up to the levels corresponding to Wistar rats. Ophthalmoscopic examinations confirmed that SkQ1 supplementation prevented development retinopathy in OXYS rats. We proposed that the beneficial effects of SkQ1 on retinopathy may be connected with reparation the mitochondrial function in the retina of OXYS rats and normalization of the level of protein CryzB. We can conclude that the SkQ1 is a perspective drug to prevention and treatment of AMD.

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**SW03.S14–43**

**The cytochrome c forms a complex with cardiolipin in a form of hydrophobic nanospheres***

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There are two basic mechanisms of cell death of a living organism: death from the direct violations of the vital functions of cells (necrosis) and programmed death caused by distress signal, received from outside or inside the cells (apoptosis). In most cases, the lipid peroxidation (LPO) is the trigger of apoptosis. LPO reactions, in its turn, are catalyzed by the complex of cytochrome c(CytC) with anionic lipids, first of all, with cardiolipin (CL) abundant in inner mitochondrial membranes(Kagan, V.E. et al. NatureChem. Biol., 2005: 1: p. 223–232). The structure of Cyt-CL complex and the mechanism of its catalytic action are, however, a matter of discussion.

In our studies it was shown that in the presence of phospholipid cardiolipin, the protein cytochrome c forms a water-insoluble precipitate (Cyt-CL complex). The cardiolipin-to-cytochrome c molar...
ratio evaluated by centrifugation and spectrophotometry was found to be constant at each given situation, the lipid to protein molar ratio being different at different pH, varying from 11 at pH 3.5–60 at pH 7.4. According to small angle X-ray scattering data, the precipitate has a microcrystalline structure with interplanar spacings characterizing the crystal unit cell equal to 11.1 ± 1.0 nm and 8.0 ± 0.7 nm; the contribution of these two structures depended on pH. These data indicate that Cyt-CL is a hydrophobic nanosphere with a diameter of about 11 nm at neutral pH and 8 nm at pH 3–5, where the cytochrome c is placed in the sphere center, completely covered with the polar heads of molecules of cardiolipin, whose tails form the outer layer of the sphere. The volume of cytochrome c globule increases in the complex as compared to free cytochrome c in the aqueous phase that is manifested in the increase of tyrosine and tryptophan fluorescence. The natural conformation of cytochrome c is disturbed, and the protein acquires a new property — the peroxidase and lipid peroxidase activities.

SW03.S14–44
Cyt-CL complex: Peroxidase activity and role in lipid peroxidation
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Cytochrome c is known to form a complex with cardiolipin (Cyt-CL), which modifies an active center of the protein. The formation of this complex plays a key role in the development of apoptosis mostly due to a new enzymatic properties of cytochrome c. It was proved that this complex can catalyze reaction of hydroxyl peroxide (acts as a peroxidase) and induce the lipid peroxidation, which is an early step of apoptosis. The mechanism of catalytic action of Cyt-CL is, however, a matter of discussion.

In our studies, the kinetics and mechanism of the peroxidase reactions catalyzed by Cyt-CL were studied by experimental measurements and mathematical simulation of the kinetics of chemiluminescence using two systems: (i) Cyt-CL + H₂O₂ + luminol (as a substrate) and (ii) Cyt-CL + hydrogen peroxide + unsaturated fatty acid (substrate) + coumarin dye C-525 (a chemiluminescence enhancer). It was shown that Cyt-CL works like all other peroxidases, with the reaction circle being composed of, at least, four particular reactions. During the interaction of Cyt-CL with lipid hydroperoxides lipid radicals are found to be produced, which are thought to initiate chain lipid peroxidation. This reaction is crucial for Cyt-CL lipoxigenase action, the first event in apoptosis development.

We investigated the regulation of Cyt-CL activity by antioxidants. It was shown that antioxidants inhibited peroxidase action of Cyt-CL in micromolar concentrations, whereas for inhibiting lipid peroxidation we needed antioxidants in higher concentrations.

SW03.S14–45
Inhibition of mitochondrial glycerol-3-phosphate dehydrogenase by alpha-tocopheryl succinate
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α-Tocopheryl succinate (TOS), an esterified analogue of vitamin E, can suppress cell growth in a number of experimental cancers. It was accepted that TOS as an inhibitor of Complex II activity of the mitochondrial respiratory chain could be a suitable drug for cancer therapy. Our data indicate that also mitochondrial glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.99.5.) is inhibited by TOS. Mammalian FAD-dependent GPDH is markedly expressed only in several mammalian tissues, such as brown adipose tissue (BAT), pancreas, plaetema and in some cancer cells. It is also well known that glycerol-phosphate shuttle has a key role in organs where glycolytic ATP production has an important role in cell energy metabolism. Therefore our aim was to compare TOS inhibitory effects on glycerol-3-phosphate (GP) and succinate (SUC) oxidases and to localize the site of TOS inhibition. As a model for our measurements we have chosen BAT mitochondria where both GPDH and succinate dehydrogenase (SDH) are very active. We evaluated TOS inhibitory effect on oxidases (include all respiratory chain components — acceptor oxygen), oxidoreductases (include particular dehydrogenases, CoQ and complex III — acceptor cytochrome c) and dehydrogenases with artificial two- (CoQ₄ and DCPIP) and one- (ferriyancide) electron acceptors. We showed the more pronounced inhibition of GP-dependent oxygen consumption by TOS than SUC-dependent one (50% inhibition at 25–50 μM TOS vs. 100–150 μM TOS, respectively). Similar results were also obtained in assays of GP and SUC cytochrome c oxidoreductases, CoQ₄, DCPIP and ferriyancide dehydrogenases. On the basis of our data we may conclude that TOS inhibition of GPDH is more pronounced than inhibition of Complex II and that the inhibitory effect for both substrates is exerted on particular dehydrogenases activity. GPDH inhibition might be important, because it is well known that neoplastic cells are highly dependent on glycolytic ATP production and GP shuttle could maintain the high rate of glycolysis by reoxidation of cytosolic NADH and avoid thus cell acidification when NAD is oxidized by lactate generation from pyruvate.

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SW03.S14–46
Bioenergetics in the cell line derived from Hurthle carcinomas
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The cell line XTC.U1 has been established from a metastasis of human Hurthle cell carcinomas (Zielke et al. 1998, Thyroid. 8: 475–483) which are characterized by abundance of mitochondria in the cytoplasm. Furthermore, the enzymatic activities of complex I and III of the respiratory chain are decreased due to mitochondrial DNA mutation in the gene encoding ND1 subunit of complex I and cytochrome b, respectively (Bonora et al. 2006, Cancer Res. 66: 6087–6096). The aim of our study was to extend the knowledge on some parameters of the bioenergetics in the oncocytic carcinoma cell line XTC.U1, using a non-oncocytic cell line TP-C1 as a control. In the cultured cells the activity of citrate synthase was measured spectrophotometrically. Safranine was used as a fluorescent probe for the evaluation of mitochondrial membrane potential. To assess oxidative stress, the content of the thioarbituric acid reactive substances (TBARS) was detected by fluorometry. We observed that the cultured XTC.U1 cell line grows in the same medium more slowly than the control TP-C1 cells. We confirmed higher amount of mitochondria by higher activity of citrate synthase in the cell line XTC.U1. We also found that in the presence of NADH-dependent substrates XTC.U1 cells had lower mitochondrial membrane potential than the control cells TP-C1 which is in agreement with the defect of complex I. Moreover, markers of oxidative stress are increased in XTC.U1 cells as compared with TP-C1 cells. We conclude that lower mitochondrial membrane potential and increased oxidative stress could contribute to the slower growth of XTC.U1 cells.

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Expression patterns of ATP-synthase subunits in liver and muscle during rat prenatal and early postnatal development

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The mitochondrial biogenesis is important for appropriate energy production during fetal growth and early postnatal adaptation to extrauterine conditions. The crucial stage of development is postnatal switch of glycolytic to oxidative metabolism. The key OXPHOS enzyme is F$_{o}$F$_{1}$-ATP synthase (complex V) producing the majority of ATP in mammals.

The aim of this study was to characterize the expression of complex V subunits – Atp5g2, Atp5a1 (nuclear genes) and Atp6 (mitochondrial gene) in rat liver and muscle during perinatal development. Moreover the expression of complex V assembly factors (Tmem70 and Atpaf2) was analyzed.

The set of 48 rat liver and 21 muscle samples was collected between 16th fetal and 18th postnatal day with adult control. Proteins and RNA were simultaneously isolated by TriReagent (MRC). The quantification of mRNA was realized by qPCR (TaqMan sonds). The dendrogram analysis of gene expression was accomplished by programmes STATISTICA 10 and Genex. Western blot data were analyzed by Quantity One. Expression was normalized to Psmb6 and Hprt mRNA or protein Hprt.

In dendrograms of mRNA patterns were found interesting tissue specific expression profiles. Transcript levels of assembly factors were significantly increasing during perinatal development in liver and decreasing in muscle. Also Atp5g2 mRNA level was significantly changed in liver however it was without significant shift in muscle. Both Atp5a1 mRNA expression pattern and protein content show significant increasing toward to birth in liver. The protein content of Atp5a1 reached major expression on 22th day of postnatal development and its transcript level does so on 1st day after birth. On the other hand – Atp5a1 expression in muscle tissue was increasing continuously during development, but protein content was reduced.

We found the tissue specific expression patterns of ATP-synthase subunits between 16th fetal and 18th postnatal day of rat development. Both Atp5a1 mRNA and protein patterns correlate in liver, but have different flow in muscle. Analyzed expression patterns may reflect adaptation changes in process of transition to extrauterine conditions.

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Mitochondrial Lace1 ATPase

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Lace1 (Lactation elevated 1) with predicted molecular weight of 55 kDa is a human homolog of yeast Afg1 (ATPase family gene 1) ATPase with sequence identity ranging from 41.5% H. sapiens vs. S. cerevisiae to 88.9% H. sapiens vs. M. musculus. The protein consists of ATP/GTP binding P-loop motif and a common five domain structure. Lace1 is predicted to be an adaptor protein of mitochondrial proteases. It has a predicted ATPase activity and shows high level of expression in active mammary gland, myocardium, kidney and liver tissue. LACE1 gene was shown to contain estrogen receptor binding sites.

The aim of our work was to perform detailed cell biological characterization of human Lace1 utilizing stable shRNA RNA interference approach, proteomics and expression analyses using human embryonic kidney cell line (HEK293).

We found that human Lace1 is a mitochondrially localized membrane-associated protein with an apparent molecular weight of 50 kDa. The prepared stable shRNA LACE1 knockdown HEK293 cell line showed markedly reduced Lace1 protein levels (<20% of controls). Using SDS-PAGE western blotting we demonstrated markedly elevated levels of subunits ND1 and Ndufb6 of respiratory complex I, SDHA of respiratory complex II and Cox2, Cox3, Cox4, Cox5a and Cox6aL of respiratory complex IV. On the other hand, the steady state level of the antiapoptotic factor Bcl-2 was found to be decreased in these cells. Furthermore, slightly elevated levels of the tumor suppressor p53 were identified in LACE1 knockdown cells. Quantitative 2D-PAGE analysis coupled to mass spectrometric identification (MS) further showed elevated accumulation of F1-alpha subunit of ATP synthase.

To summarize, our work have thus far identified Lace1 as a mitochondrial factor likely playing a role in protein turnover of subunits of the oxidative phosphorylation system, confirming its predicted function as an adaptor protein of mitochondrial proteases.

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References


Voltammetry of the cytochrome c-cardiolipin complex in the immobilized state. Implications in apoptosis initiation

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Cardiolipin (CL) is a phospholipid component of the mitochondrial inner membrane where it is synthesized from phosphatidylglycerol and cytidinediphosphate-diacylglycerol. Its oxidation upon complex formation with cytochrome c (cytc) plays a crucial role in triggering apoptosis. It has been shown that both the ferri- and ferrous forms of the heme group of a CL:cytc complex exist as multiple conformers at a physiological pH [1]. The cytc-CL interaction occurs at the electrostatic interface between the phosphate groups of CL and some positively charged residues on the protein surface, as the lysines 72, 73, 86. Moreover, insertion of one of the acyl chains deep into the protein leads to a conformational change resulting in the swapping of the axial methionine 80 by a lysine residue [1,2,3]. In this study, we have investigated by means of voltammetry and fluorescence the cytc-CL complex formed by the wild type protein and the triple mutant K72A/K73A/K79A, in which most of the lysines involved in the interaction have been suppressed. To mimic the in vivo conditions, the CL:cytc complex has been adsorbed onto a gold electrode functionalized with an hydrophobic SAM made of decanethiol. Among the main results, an electrocatalytic behavior of the complex towards the O$_2$ molecule has been detected.

Mitochondrial Lace1 ATPase

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Lace1 (Lactation elevated 1) with predicted molecular weight of 55 kDa is a human homolog of yeast Afg1 (ATPase family gene 1) ATPase with sequence identity ranging from 41.5% H. sapiens vs. S. cerevisiae to 88.9% H. sapiens vs. M. musculus. The protein consists of ATP/GTP binding P-loop motif and a common five domain structure. Lace1 is predicted to be an adaptor protein of mitochondrial proteases. It has a predicted ATPase activity and shows high level of expression in active mammary gland, myocardium, kidney and liver tissue. LACE1 gene was shown to contain estrogen receptor binding sites.

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We found that human Lace1 is a mitochondrially localized membrane-associated protein with an apparent molecular weight of 50 kDa. The prepared stable shRNA LACE1 knockdown HEK293 cell line showed markedly reduced Lace1 protein levels (<20% of controls). Using SDS-PAGE western blotting we demonstrated markedly elevated levels of subunits ND1 and Ndufb6 of respiratory complex I, SDHA of respiratory complex II and Cox2, Cox3, Cox4, Cox5a and Cox6aL of respiratory complex IV. On the other hand, the steady state level of the antiapoptotic factor Bcl-2 was found to be decreased in these cells. Furthermore, slightly elevated levels of the tumor suppressor p53 were identified in LACE1 knockdown cells. Quantitative 2D-PAGE analysis coupled to mass spectrometric identification (MS) further showed elevated accumulation of F1-alpha subunit of ATP synthase.

To summarize, our work have thus far identified Lace1 as a mitochondrial factor likely playing a role in protein turnover of subunits of the oxidative phosphorylation system, confirming its predicted function as an adaptor protein of mitochondrial proteases.

This work was supported by First Faculty of Medicine, Charles University in Prague, by Grant Agency of the Charles

References

Inhibiting the activity of mitochondrial ATP synthase with Oligomycin-A suppresses motility and ‘in vitro’ capacitation achievement of boar spermatozoa but does not affect the sperm energy levels

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In this work we have studied the role of mitochondrial ATP synthase on the function and survival of mammalian spermatozoa. With this aim, boar spermatozoa were incubated in a defined capacitation medium with or without Oligomycin-A, a specific inhibitor of the F0 component of the mitochondrial ATP synthase, for a 4-h period at 37.5°C and 5% CO2. After incubating samples for 4 h, 10 μg/ml progesterone was added to induce acrosome exocytosis. Sperm motility, sperm viability, membrane lipid disorder, intracellular calcium levels, acrosome exocytosis, O2 consumption and ATP levels were evaluated at 0 h, 2 h and 4 h, and 5 min, 30 min and 1 h after adding 10 progesterone. Oligomycin-A resulted to reduce the sperm motility and inhibit the ability of boar spermatozoa to undergo capacitation-related changes, as different sperm parameters showed. In contrast, rhythm of O2-consumption, intracellular ATP levels or mitochondrial membrane potential were not affected by the presence of Oligomycin-A in the capacitation medium. When progesterone was added, and as expected, fast and intense peaks in O2 consumption and ATP levels were observed in the absence of Oligomycin-A. In contrast the presence of Oligomycin-A resulted to inhibit the progesterone-induced acrosome exocytosis, and the corresponding peaks of O2 consumption and ATP levels were not observed. In conclusion, despite mitochondrial ATP synthase activity appearing to play a main role in the management of boar sperm motility, capacitation and acrosome exocytosis, it does not seem to be linked to changes in the overall maintenance of adequate energy levels in stages other than acrosome exocytosis.

Clusterin action on mitochondrial dynamics in insulin-secreting beta-cells

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Mitochondrion is a dynamic cellular organelle showing a diverse morphological change in response to biological processes including energy metabolism and viability of the cells. Clusterin is an intriguing protein with a variety of biological functions, particularly in association with cytoprotection. Pancreatic beta cells produce and secrete insulin, but are susceptible to internal and external challenges, leading to hyperglycemia and diabetes. The present study aimed to elucidate the clusterin action on the processes of beta cell mitochondria regarding to mitochondrial dynamics including their fusion/fission and autophagy formation.

Clusterin expression was regulated from by transfection of overexpression vector and siRNA, respectively using MIN-6 and INS-1 cell lines which secrete insulin as well as clusterin simultaneously. Mitochondrial proteins which are associated with mitochondrial fusion and fission were monitored after clusterin over-expression and deletion, along with their morphological observation. We found that clusterin overexpression down-regulated the mitochondrial fusion factors including OPA-1, MFN-1, MFN-2. In contrast, the mitochondrial fusion proteins were up-regulated by knock-down of the clusterin genes. Although there was no significant change in mitochondrial fission proteins (Drp-1 and-2), LC3B, an autophagy marker, was decreased by clusterin over-expression, while being increased by clusterin deprivation. We have traced the expressions of OPA-1 and LC3B in the mitochondria and lysosomes by confocal microscopy, demonstrating a concurrent modification in the cytoplasm of the bet cells. We also found a significantly reduced autophagosomes upon overexpression of clusterin, and their increased formation by downregulation of the clusterin.

Taken together, these results suggest that clusterin is involved in cellular functions and viability by modulating mitochondrial dynamics, particularly by suppression of the mitochondrial fusion as well as inhibition of autophagy.

A Ubiquitin-dependent protein quality control system at the plasma membrane

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Down-regulation of cell surface receptors and transporters is mediated by a series of membrane trafficking steps including ubiquitin-mediated endocytosis, ESCRT-mediated cargo(Ub) recognition, sorting and packaging into vesicles that bud into the lumen of the endosome (MVBs), and endosome-lysosome fusion that delivers sorted receptors into the lumen of the lysosome where degradation occurs. Failure to attenuate growth-factor receptor signaling at the plasma membrane (PM) by endocytic down-regulation can lead to cancer. Maintenance of proper PM proteostasis, particularly with respect to ion channels and nutrient transporters, is crucial to prevent loss of PM integrity and dissipation of essential ion and chemical gradients. As such, when PM resident proteins become damaged or misfolded, they must be recognized, removed by endocytosis and delivered to the lysosome for degradation. Thus, cells must maintain a ‘cradle to the grave’ quality monitoring system for integral membrane proteins, yet the mechanisms of quality surveillance, particularly at the PM, remain poorly understood. We have evidence that the E3 ubiquitin ligase Rsp5, the yeast homolog of Nedd4, is a key mediator of protein quality control at the PM. We show that proteotoxic stress triggers global activation of Rsp5-dependent ubiquitination, endocytosis, and vacuolar trafficking of PM proteins, and that yeast mutants attenuated for this response are highly sensitive to proteotoxic stress. This stress-induced surface remodeling is mediated by a family of Rsp5 adaptors known as artesin-related trafficking adaptors (or ARTs), which target Rsp5 ubiquitin ligase activity to specific PM proteins during proteotoxic stress. The ART1 protein undergoes a phosphorylatory cycle. We propose that the ubiquitin-mediated ART-Rsp5 PM surveillance system
SW03.S15–2
New genes involved in peroxisomal and soluble protein fructose-1,6-bisphosphatase autophagic degradation in yeasts
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Pichia pastoris and Hansenula polymorpha yeasts belong to the most convenient models for studying mechanisms of selective and non-selective autophagic degradation of cellular material. We described recently the role of Ccz1, Ypt7, and Mon1 proteins in autophagic pathways in P. pastoris. Strains with a deletion of either of these genes displayed defects in autophagy and pexophagy and biosynthetic Cvt pathway. These proteins act in regulation of pexophagy and biosynthetic Cvt pathway. Gss1 of H. polymorpha shows high homology level with the expression of FBP protein (FBP) in S. cerevisiae. It was shown that FBPase is mostly degraded in the presence of glucose, and notype and glucose catabolite repression defect. High and low glucose concentrations, pexophagy deficiency phenotype and glucose catabolite repression defect.

Some cytosolic proteins such as gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) could be specifically degraded during shift from gluconeogenic substrates to glucose. In S. cerevisiae, such degradation involves selective autophagy pathway. In the methylotrophic yeasts FBPase additionally participates in methanol utilization pathway. Mechanisms of catabolite degradation of FBPase in methylotrophic yeasts has not been studied so far.

The FBPases of P. pastoris and H. polymorpha strains were analyzed by Western blot using the antibodies for FBP protein of S. cerevisiae. It was shown that FBPase is mostly degraded after 5 h of incubation of cells cultivated in gluconeogenic substrates in glucose-containing medium. After shifting the cells from methanol or ethanol-containing media on glucose medium, FBPase activity of P. pastoris and H. polymorpha wild-type strains decreased 4.5–5 and 2–2.5 times, respectively. To study the mechanisms of catabolite degradation the recombinant strains with the expression of FBP fused with GFP under the control of AOX and FBP promoters were constructed and analyzed. FBPase degradation in the P. pastoris mutants defective in Gss1, Ccz1, Ypt7, and Mon1 is under investigation.

SW03.S15–3
Ubiquitination is not required for proteasome-mediated degradation of myelin basic protein
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Degradation of Multiple Sclerosis (MS) autoantigen myelin basic protein (MBP) by enzymes was extensively studied during decades as an important component of autoimmune response in MS. In the present study we addressed a question if MBP is a substrate for proteasome-ubiquitin machinery and how ubiquitination system controls its degradation. Our data suggest that 26S-mediated degradation of intracellular MBP is ubiquitin-independent. We showed that the most unusual feature of this autoantigen, namely its drastic basicity, is responsible for direct interaction of MBP with 19S regulatory particle. ‘Naturally unfolded’ conformation of MBP enhances its swallowing by 26S proteasome upon binding to Rpn10 subunit. In contrast to antizyme-mediated proteasomal ODC protein degradation, mechanism discovered in the present study may have general fundamental significance for a broad cluster of highly positively charged intrinsically disordered proteins, which may probably be hydrolyzed by the proteasome machinery bypassing ubiquitin.

SW03.S15–4
Brain-derived immunoproteasome generates increased amounts of encephalitogenic MBP peptide epitope
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Triggering of autoimmune diseases by autoantigens is poorly understood. One of the crucial steps in antigen presentation on MHC 1 is degradation of antigenic protein by proteasome. We studied proteasome-mediated degradation of myelin basic protein (MBP), one of major autoantigens in multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). We have demonstrated a dramatic shift in the balance between constitutive and immune proteasomes in the CNS of SJL mice with EAE, with immunoproteasome subunit LMP2 localized mainly in oligodendrocytes. Patterns of MBP degradation by proteasome from brain of EAE mice and control non-treated mice were compared by MS spectra using 16O/18O labeling and isotope-labeled synthetic peptides. Elevated levels of immunoproteasome in brain of mice with EAE result in an increased production of several peptides, including peptide ENPVHFF, a part of encephalitogenic MBP region. Peptidyl epoxidekine inhibitor of LMP2 immunoproteasome subunit affects catalytic activity of brain-derived immunoproteasome in vitro and ameliorates ongoing demyelination in vivo, suggesting a novel treatment modality of autoimmune neurological diseases.

SW03.S15–5
Basic charge rather than polyubiquitination is sufficient for proteasomal degradation of the myelin basic protein
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Here we present distinct molecular mechanism of proteasome-mediated and ubiquitin-independent hydrolysis of myelin basic protein (MBP). Firstly, we showed that ubiquitin receptor of 19S regulatory particle Rpn10 might directly bind MBP. This interaction seems to lack the involvement of any specific determinants in MBP molecule and generally is mediated by its surface charge.
Strength of binding of MBP to Rpn10 is estimated as $K_d = 2-3 \mu M$, which is at least one order higher than Ub$_4$-Rpn10 interaction. We suggest that interaction of MBP with Rpn10 results in complete unfolding of MBP. Because Rpn10 is located near the entrance to the proteolytic chamber, C- or N-terminus of unfolded MBP may be rapidly uptaken by proteasome and stretched through the pore of Rpt1-6 ring and whole protein further is hydrolyzed in proteolytic chamber. Several post-translation modifications of MBP molecule including deamination were documented in number of studies devoted to MS. Our observations suggest that MBP retains as a proteasome substrate only bearing high positive charge. We speculate that, leading to loss of MBP in general, MBP deimation may partially act as self-protection system, preventing MBP proteolysis by proteasome as deaminated MBP is completely invisible for the 26S proteasome.

**SW03.S15-6**

**Structural study of ubiquitin-like domains: towards protein interactions in ubiquitin proteasome system**

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Cell homeostasis is maintained via strictly regulated processes in which controlled protein degradation plays an important role. Proteins destined for degradation are posttranslationally modified with polyubiquitin chains and targeted to proteasome via Ubiquitin Proteasome System (UPS). Moreover, ubiquitination is emerging as a general device for controlling many other cellular functions.

UPS involves ubiquitination of target substrates via set of enzymes, followed by substrate transfer and degradation in the 26S proteasome. Target substrates are recognized either directly by (poly)ubiquitin receptors or indirectly by ubiquitin-like domain (Ubl) protein shuttles. This study focuses on the structural features of proteasome-Ubl recognition of the DNA damage-inducible protein (Ddi), a member of protein shuttles from budding yeast. Ddi harbors typical two-domain architecture of N-terminal ubiquitin-like and C-terminal ubiquitin associated domain that binds polyubiquitinated chains. Moreover, it possesses a highly conserved retroviral protease-like domain with putative catalytic function.

Ubl domains of the yeast Ddi1 and its human ortholog Ddi2 were cloned, isotopically expressed and purified in a sufficient yield for NMR spectroscopy analysis. The structures of both Ubls were solved and compared with solution structures of the mouse ortholog Ddi1-Ubl and human ubiquitin.

**SW03.S15-7**

**Search of proteolytical activity of DNA damage-inducible protein**

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In a strictly regulated process of maintaining cell homeostasis, one of its very important pathways is controlled protein degradation carried out by Ubiquitin Proteasome System (UPS). Protein degradation via this pathway consists of series of enzymatic reactions of labelling target substrates by ubiquitin molecule, followed by transfer of the ubiquitinylated complexes to the proteasome and their subsequent proteolysis by the 26S proteasome machinery. One of the possible way of transferring ubiquitin-labelled proteins to the proteasome exploits so called proteasome shuttling proteins, molecules possessing at least two-domain architecture including domain interacting with ubiquitin as well as domain binding to the proteasome.

DNA damage-inducible protein 1 (Ddi1) from the budding yeast belongs to the family of proteasomal shuttles. More interestingly, Ddi1 possesses also retroviral protease-like (RVP) domain located in central part of the protein sequence. Ddi1 is conserved throughout all sequenced eucaryotic species with RVP domain being the most conserved part of the protein. Structural fold of Ddi1 RVP domain resembles the 3D structure of proteases from retroviruses, bearing also characteristic catalytical triad DT[S]G located in the putative active site which is also well conserved throughout eukaryotic species.

In this project the putative proteolytic activity of Ddi1 proteins was investigated. The orthologs of both the full-length and truncated (RVP only) Ddi1 protein from human, yeast and Leishmania were cloned, expressed in *E. coli* and purified. The recombinant proteins were used for protease cleavage assays using N-terminomic Proteomic Identification of protease Cleavage Sites (PICS) and proteolysis of natural substrates of retroviral aspartic proteases. The results of these analyses will be discussed and compared to the substrate specificity of closely related HIV protease.

**SW03.S15-8**

**The effect of beclin-1, LC3 II/I, BCL-2 and phospho BCL-2 in hypercholesterolemia induced oxidative stress**

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Hypercholesterolemia is the major risk factor for atherosclerosis and the development of cardiovascular diseases such as heart failure. Increased reactive oxygen species (ROS) production and the resulting oxidative cell stress has been shown to induce autophagy in many disease. Ox-LDL alters the activity of the autophagy through the LC3/beclin-1 pathway. When the proteasome is impaired, autophagy provides a possible alternate pathway for clearing aggregated proteins. Autophagy is a accepted to be a housekeeping process which is essential in the heart and brain.

Incidentally, the ratio of the protein expression of membrane associated LC3-II to cytosolic LC3-I (LC3II:I), is indicative of autophagosome formation, and therefore is often used to assess autophagic activity. In connection with the ROS induced autophagy through a beclin-1 dependent pathway, is associated with autophagic induced cell-death. Beclin-1 is negatively regulated by its interaction with the anti-apoptotic protein Bel-2 under normal conditions. However, increased ROS activates the ubiquitin-proteasome system, which functions to degrade Bel-2. This allows for beclin-1 activation subsequently resulting in autophagic cell death. It has revealed that free cholesterol induces a time dependent upregulation of microtubule-associated protein-1 light chain 3-II (LC3-II) and ox-LDL activates the autophagic lysosome pathway through the LC3/beclin1. This results an increase in the formation of autophagosomes and autolysosomes, leading to the degradation of ox-LDL.

In the present study to elucidate in vivo role of autophagy for hypercholesteremic rabbits and the effects of Vitamin E, we investigate MDA and vitamin E levels from blood and LC3 II/I, beclin-1, Bel-2, phospho Bel-2 protein levels in heart tissues for compared them to each other and the effects of vitamin E on these changes in the atherosclerotic rabbit model induced by 2% cholesterol containing diet was planned. The results will be discussed.

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The injured myocardium represents an environment in which there are various factors that promote cell apoptosis, such as oxidative stress, hypoxia and inflammatory reactions. High cholesterol raises important for cardiac dysfunction, heart attack, and stroke. Oxidative stress describes an imbalance between antioxidant defense and the production of reactive oxygen species (ROS), increased reactive oxygen species (ROS) production and the resulting oxidative cell stress has been shown to cell death/apoptosis, mitochondrial dysfunction, cardiac remodeling, and dysfunction. Process of apoptotic cell death shown that changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Bcl-2 family proteins such as Bcl-2, Bcl-XL, Bax, Bak, Bad and Bid are central regulators of cell life and death. Caspases are a family of cysteine protease that play essential roles in apoptosis which are cleavage some of the final targets such as lamins ICAD/DFS45 (inhibitor of caspase activated DNase or DNA fragmentation factor 45) PARP (poly-ADP ribose polymerase). Apoptosis inducing factor is involved in initiating a caspase-independent pathway of apoptosis. Caspases 3-9, apoptosis inducing factor (Aif) and Bax induce cell death, on the contrary to Bcl-2 trigger cell survival.

In our experiment 20 male albino rabbits (1–2 months old) were assigned randomly to four groups. The first group of rabbits, the control rabbits, was only fed with diet. The second group was fed with diet containing 2% cholesterol, third group were assigned randomly to four groups. The first group of rabbits was fed with diet containing 2% cholesterol, second group was fed with diet containing 2% cholesterol and received injections of 50 mg/kg/day of vitamin E intramuscularly and the rabbits in the fourth group were fed with diet containing 2% cholesterol and received injections of 50 mg/kg/day of vitamin E intramuscularly. After 8 weeks; MDA and vitamin E in blood was detected by HPLC. Apoptosis of heart tissue showed by DNA fragmentation. The left ventricle of heart tissues were removed and protein levels of Bcl-2, Bax, apoptosis inducing factor (Aif), Caspase-9 and Caspase-3 were measured. The results indicate role of apoptotic process for hypercholesterolemic rabbits will be discussed.

SW03.S15–9
High cholesterol diet induced apoptotic process on rabbit cardiac myocyte failure
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SW03.S15–10
Proteomic study of linear polyubiquitin chains interactome
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Ubiquitylation, being one of the most widespread and important post-translational modifications of eukaryotic proteins, regulates a multitude of critical cellular processes both in normal and pathological conditions. Ubiquitin can be attached to a target protein either as a monomer or in a form of polyubiquitin chains of various structure. To date little is known about the specificity of recognition of different chain species and their involvement in cell metabolism. It is also unclear how cell machinery discriminates between polyubiquitin chains of the same architecture, but various lengths. Therefore identification of interaction partners for these chains will help to reveal their roles in the cell and the mechanisms of their recognition. To meet this goal we identified proteins that specifically recognize linear polyubiquitin chains of various lengths and therefore may serve as ubiquitin receptors in downstream signaling cascades. The approach employed was based on a pulldown of ubiquitin receptors from cell lysate. Tagged mono-ubiquitin and linear ubiquitin chains in the form of di- and tri-ubiquitin were used as baits to fish out interaction partners from HeLa cell lysate. The obtained proteins were subsequently identified by mass spectrometry analysis. The selected targets were validated by Western blotting.

The identified protein set is rather diverse and comprises enzymes from ubiquitylation cascade, known ubiquitin receptors and novel interactors. Proteins from the last group lack any known ubiquitin-binding domains and represent the most interesting targets for further studies. The results of this proteomic screening shed new light on the specificity of polyubiquitin chains recognition and suggest new roles of ubiquitin in cell metabolism, including possible involvement into RNA splicing and processing pathways.

The reported study was partially supported by SIFPOD-SEMM fellowship and a grant from Russian Foundation for Basic Research (project 12-04-32136).

SW03.S15–11
Autophagosomal Syntaxin17-dependent lysosomal degradation maintains neuronal function in Drosophila
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During autophagy, phagophores capture portions of cytoplasm and form double-membrane autophagosomes to deliver cargo for lysosomal degradation. How autophagosomes gain competence to fuse with late endosomes and lysosomes is not known. Here we show that Syntaxin17 is recruited to the outer membrane of autophagosomes to mediate fusion through its interactions with ubiquitin (SNAP-29) and VAMP7 in Drosophila. Loss of these genes results in accumulation of autophagosomes and a block of autolysosomal degradation during basal, starvation-induced and developmentally autophagy. Viable Syntaxin17 mutant adults show large-scale accumulation of autophagosomes in neurons, severe locomotion defects and premature death. These mutant phenotypes cannot be rescued by neuron-specific inhibition of caspases, suggesting that caspase activation and cell death do not play a major role in brain dysfunction. Our findings reveal the molecular mechanism underlying autophagosomal fusion events, and show that lysosomal degradation and recycling of sequestered autophagosome content is crucial to maintain proper functioning of the nervous system.

SW03.S15–12
Dual degradation in G1 and S/G2 phases limits Stem Loop Binding Protein expression to the S phase
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Expression of replication-dependent histone mRNAs is tightly regulated during cell cycle and much of the regulation is at posttranscriptional level. Stem Loop Binding Protein (SLBP) binds to 3′
end of histone mRNA and required for several steps of histone mRNA metabolism including pre-mRNA processing, translation and regulation of mRNA stability. SLBP expression is limited to S phase and this is one of the major mechanisms that restricts histone mRNA biosynthesis thus histone production to the S phase. At the end of S phase SLBP is rapidly degraded due on double phosphorylation on Thr 60 and Thr 61 by CK2 and CyclinA/Cdk1 respectively. SLBP is kept low in G1 and rapidly accumulates at the beginning of S phase without significant change in mRNA level. Previously, it was reported that the translation rate of SLBP mRNA at early G1 is low, leading to the reduced expression of SLBP. Here we propose proteasome mediated degradation as an additional posttranslational regulation that keeps SLBP expression low during G1 phase and this degradation is independent of previously identified degradation mechanism at the end of S phase. By treating synchronized cells with Cycloheximide we compared the stability of SLBP in different phases of cell cycle and found that similar to S/G2, the stability of SLBP is low at mid G1 compared to S phase. Further, treatment of cells with proteasome inhibitor prevented degradation and increased the SLBP level in G1. In order to see whether this is the same degradation mechanism as in S/G2, we checked the stability of S/G2 degradation mutant SLBP (SFT60A61P) in G1 and showed that it is also degraded similar to endogenous SLBP. Our findings show that restriction of SLBP expression to S-phase is ensured by several distinct mechanisms in order to limit histone production to S phase.

**Characterization of a deubiquitylation gene in Drosophila melanogaster**

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Removal of ubiquitin from poly-ubiquitylated proteins is performed by deubiquitylating enzymes (DUBs) that catalyze the cleavage of isopeptide bonds between target proteins and ubiquitin. Although the study of DUBs intensified in the last few years, understanding of their functions remains considerably limited. Genetic analysis of mutant phenotypes in the well-characterized model organism, *Drosophila melanogaster* can provide important information to elucidate the function of DUBs.

RNAi knockdown of one of the DUB genes coding for *Drosophila* ortholog of human Usp5 (*DmUsp5*) causes early pupal lethality. Late lethality of these animals is accompanied by an increase in the number apoptotic cells in the larval brain and imaginal discs. Null alleles of *DmUsp5* were established by P element remobilization. The development of the homozygous null animals stops in L3 and they die in this stage after a 5 day long stagnation period. Acridine orange staining of L3 larval brains and wing discs revealed a very high incidence of apoptosis in these animals. In addition to this, the expressions of *reaper* and *hid*, but not *grim* pro-apoptotic genes have been elevated in the *DmUsp5* mutant larval brains and imaginal discs. A heterologous complementation experiment confirmed functional homology between *DmUsp5* gene and yeast Ubp14. In addition to this, we also show that free polyubiquitin chains accumulated in *DmUsp5* mutants similarly to the yeast Ubp14 mutants that further support functional conservancy.

Based on these observations we identified the evolutionarily conserved Usp5 in *Drosophila* and demonstrated its involvement in regulating apoptosis.

**Two isoforms of Hsp70 nucleotide exchange factor Fes1 are essential for compartment-specific proteasomal degradation of misfolded proteins**

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Protein folding stress results in the generation of aggregation-prone misfolded polypeptides that have a strong negative impact on cells. Cellular defense mechanisms recognize and degrade misfolded proteins by employing Hsp70 molecular chaperones that directly can bind hydrophobic segments of proteins. During our studies of Hsp70 function in the yeast cells, we have uncovered that the Hsp70 nucleotide exchange factor Fes1 promotes the proteasomal degradation of misfolded proteins associated with Hsp70. Intriguingly, our analysis show that the *FES1* transcript undergoes alternative splicing resulting in the expression of the two functional isoforms, Fes1S and Fes1L. Both isoforms stimulate ADP release from Hsp70 with similar kinetics and complement *fes1* growth phenotypes. Strikingly, Fes1S is localized to the cytoplasm and Fes1L to the nucleus and each isoform is specifically required for the proteasomal degradation of misfolded proteins in their respective cellular compartment. Our findings support the notion that compartmentalized protein homeostasis relies on degradation pathways that are initiated by Hsp70 nucleotide exchange factors.
SW03.S15–16
Platelet PAR4 associated with neutral sphingomyelinase responsible for thrombin-stimulated ceramide-NF-kappaB signaling in human platelets
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Thrombin activates platelets mainly through protease-activated receptor (PAR)1 and PAR4. However, downstream platelet signaling between PAR1 and PAR4 is not yet well understood. This study investigated the relationship between nSMase/ceramide and the NF-κB signaling pathway in PARs-mediated human platelet activation. Human platelets stimulated by thrombin, 3-OMS (a neutral sphingomyelinase [nSMase] inhibitor) and Bay11-7082 (an NF-κB inhibitor) significantly inhibited platelet activation such as P-selectin expression. Thrombin also activated IκB kinase (IκK)β and IκBa phosphorylation; such phosphorylation was inhibited by 3-OMS and SB203580 (a p38 MAPK inhibitor). Moreover, 3-OMS abolished platelet aggregation, IκKβ, and p38 MAPK phosphorylation stimulated by PAR4-AP (a PAR4 agonist) but not by PAR1-AP (a PAR1 agonist). Immunoprecipitation revealed that nSMase was directly associated with PAR4 but not PAR1 in resting platelets. In human platelets, C24:0-ceramide is the predominant form of ceramides in the LC/MS-MS assay; C24:0-ceramide increases after stimulation by thrombin or PAR4-AP, but not after stimulation by PAR1-AP. We also found that C2-ceramide (a cell-permeable ceramide analogue) activated p38 MAPK and IκKβ phosphorylation in platelets and markedly shortened the occlusion time of platelet plug formation in vivo. This study demonstrated that thrombin activated nSMase by binding to PAR4, but not to PAR1, to increase the C24:0-ceramide level, followed by the activation of p38 MAPK-NF-κB signaling. Our results showed a novel physiological significance of PAR4-nSMase/ceramide-p38 MAPK-NF-κB cascade in platelet activation.

SW03.S15–17
The malin-lafortin complex downregulates R6, a PP1 regulatory subunit, targeting it to lysosomal degradation
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Lafora disease (LD, OMIM 254780) is a progressive myoclonus epilepsy characterized by a fatal neurological deterioration and the presence of inclusions of poor-branched glycogen, called Lafora bodies (LB). These LB suggest a disorganization in glycogen metabolism. PPP1R3D, R6, is a protein subunit of PP1 that targets the phosphatase to glycogen particle. The recruitment of PP1-R6 holoenzyme result in an enhancement of glycogen accumulation. We have studied the interaction between R6 with malin and laforin, the two proteins whose mutations cause Lafora disease. We found that laforin interacts with R6 by different techniques. As laforin could act recruiting substrates for malin to be ubiquitinated, we studied wether R6 could be a novel malin substrate. Interestingly, we found that R6 is rather mono/ubiquitinated than polyubiquitinated. This posttranslational modification of R6 acts as a signal for degradation of the protein by the autophagic pathway. Thus, the glycogenic activity of R6 is counteracted by the laforin-malin modulation. In addition, we showed a common localization of R6, malin, laforin with glycogen synthase, suggesting the formation of a subcellular structure where glycogen homeostasis is regulated. These results highlight the importance of R6 regulation and its relationship with the Lafora disease related proteins, malin and laforin.

SW03.S15–18
Discovery and application of DNA aptamers which specifically bind and inhibit WWP1 ubiquitin ligase in the osteoblast
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WWP1 ubiquitin ligase complexes with Schnurri3 to polyubiquitinate Runx2, the key transcriptional regulator of osteoblast differentiation. Inhibition of WWP1 ubiquitin ligase may thus stimulate osteoblast differentiation as a therapeutic strategy in osteoporosis sufferers. Here, we present the discovery and application of DNA aptamers that inhibit WWP1 ubiquitin ligase. Unique DNA aptamers were selected from libraries against the C-lobe of the catalytic domain of WWP1 ubiquitin ligase with a Ki of 2.0 μM and were shown to inhibit the catalytic activities of the purified enzyme. The aptamers were delivered in a cell culture system and found to migrate to the nuclear and had specific effects on the cellular phenotype. The work lays a foundation for extending the intracellular applications of DNA aptamers. This research was funded by Hong Kong UGC GRF grant HKU 777109M.

SW03.S15–19
Cystatin SN neutralizes the inhibitory effect of cystatin C on cathepsin B activity
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Cystatin SN (CST1) is one of several salivary cystatins that form tight equimolar complexes with cysteine proteases, such as the cathepsins. High expression of CST1 is correlated with advanced pTNM stage in gastric cancer. However, the functional role of CST1 in tumorigenesis has not been elucidated. In this study, we showed that CST1 was highly expressed in colon tumor tissues, compared to nontumor regions. Increased cell proliferation and invasiveness were observed in HCT116 cell lines stably transfect- ed with CST1 cDNA (HCT116-CST1), but not in CST3-transfected cells. We also demonstrated that CST1-overexpressing cell lines exhibited increased tumor growth as well as metastasis in a xenograft a nude mouse model. Interestingly, CST1 interacted with cystatin C (CST3), a potent cathepsin B (CTSB) inhibitor, with a higher affinity than the interaction between CST3 and CTSB in the extracellular space of HCT116 cells. CTSB-mediated cellular invasiveness and proteolytic activities were strongly inhibited by CST3, but, in the presence.

SW03.S15–20
Divergent mechanisms of Ran pathway organization in metazoan species
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The small GTPase Ran controls many processes of eukaryotic cells, including nucleocytoplasmic transport and mitotic spindle assembly. Asymmetrical distribution of the GDP-bound forms of Ran is maintained through the carefully controlled distribution of Ran’s regulators: Ran’s guanidine...
exchange factor is RCC1, a chromatin-bound protein that provides a high concentration of Ran-GTP within the interphase nucleus and around mitotic chromosomes. The GTPase activating protein for Ran is RanGAP1, which is localized in the cytoplasm and at the cytosolic face of the nuclear pore complex (NPC) and which maintains a low level of Ran-GTP in interphase cytosol. The mechanism of the RanGAP1 anchoring to the NPC has been well-studied in vertebrate cells. Upon conjugation to SUMO1 (Small Ubiquitin-like MODifier), RanGAP1 binds to the IR domain of the nucleoporin Nup358/RanBP2 within a stable complex that also contains the SUMO-conjugating enzyme Ubc9.

We wish to compare Ran pathway organization between different metazoan species, and particularly whether they utilize common or divergent mechanisms for localizing RanGAP1. Toward this end, we have examined the subcellular localization of RanGAP1 in the fly D. melanogaster. Drosophila has clear homologues for RanGAP1 (dRanGAP1), RanBP2 (dRanBP2) and Ubc9 (dUbc9). dRanBP2 notably lacks a sequence with obviously homology to the IR domain. Nevertheless, we found that dRanGAP1 localized at the nuclear envelope (NE) in Drosophila cells. This localization was dependent upon dRanBP2, and we could document the physical association of dRanGAP1 with dRanBP2 through reciprocal co-immunoprecipitation. On the other hand, we did not find that dRanGAP1 becomes SUMOylated, nor did its localization at the NPCs of Drosophila cells require a functional SUMO pathway.

Together, our findings suggest that anchoring of RanGAP1 to the NPC in vertebrate and invertebrate cells is mediated by different biochemical mechanisms. Our results also imply that the IR domains of mammalian RanBP2 as well as SUMOylation of RanGAP1 are vertebrate-specific adaptations. We are currently working to elucidate the complete composition of the dRanGAP1/dRanBP2 complex, as well as the essential interactions that target dRanGAP1 to the NPC.
SW04 Molecular Mechanisms of Disease

SW04.S16 Biochemistry for Medicine (IV-S16)

SW04.S16–1
Nuclear organization in lymphoid cells: implications for translocations and gene regulation
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Chromosomes are confined to chromosomal territories in the nuclear space. Translocations that lead to exchanges of chromosome arms may disrupt this cellular order and lead to relocalization of genes within the nucleus.

We have studied the localization of cyclin D1 (CCND1) and c-myc genes within the nuclei in normal human lymphocytes, mantle cell lymphoma and Burkitt lymphoma cells and cell lines. We have shown that both CCND1 and c-myc genes were relocalized form the chromosomal periphery into the central perinucleolar region following translocation. We have also shown the presence of nucleolin-binding sites in the vicinity of the CCND1 gene and show that they upregulate transcription from the CCND1 promoter. We also show intensive binding of nucleolin to the CCND1 gene promoter after the translocation and that these changes span over very large regions. The same pattern is observed in the Burkitt lymphoma, where c-myc is upregulated by nucleolin.

We propose a novel mechanism of carcinogenesis brought about by chromosomal translocations where the relocalization of genes within the nucleus.

SW04.S16–2
A corrected model of lectin pathway complement activation based on in vitro evolved protease inhibitors and kinetic studies
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The complement system is part of the immune system. It protects us against invading pathogens and dangerously altered self-cells. The system can be activated through three distinct routes, the classical, the alternative and the lectin pathway. Each pathway is triggered by specific danger signals but eventually culminate in a common effector route. Unlike the classical pathway, which relies on the existence of already developed specific antibodies, the lectin pathway provides an immediate antibody-independent defense. It is triggered when large pattern recognition proteins recognize common signatures on pathogens or altered host cells.

This leads to activation of associated MASP (mannan-binding lectin-associated serine protease)zymogens. In vitro studies showed that MASP-2 has all activities needed for igniting the pathway. It can auto-activate and cleave complement components C2 and C4 forming a centrally important cascade element, the C4b2a C3 convertase. MASP-2 had therefore been recognized as the autonomous pathway activator. MASP-1 can also auto-activate and cleave C2, but it cannot cleave C4. Consequently, MASP-1 had been recognized as an auxiliary pathway compo-

ment. We evolved a pair of unique, monospecific MASP-1 and MASP-2 inhibitors. Both inhibitors completely block the lectin pathway indicating that the genuine role of MASP-1 has been overlooked. With a series of experiments we revealed a completely novel mechanism of lectin pathway activation. We show that MASP-1 is an essential component as it is the dedicated activator of MASP-2. Since unregulated activation of the lectin pathway plays a dominant role in provoking massive tissue damage upon myocardial infarct and stroke, these inhibitors are also lead molecules for subsequent drug development.

SW04.S16–3
Immunoproperties of hypothalamic proline-rich polypeptides for humoral and adaptive immune response against methicillin-sensitive and methicillin-resistant Staphylococcus aureus
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Antibiotic resistance and frequent occurrence of nosocomial infections and sepsis remain a major threat for infected people and hospitalized patients. A recent increase of multiresistant methicillin-resistant Staphylococcus aureus (MRSA) strains have developed resistance to started to pose great difficulty in selecting antimicrobial agents. We analyzed the impact of hypothalamic neuropeptide PRP-1 also known as galarmin on adaptive immune response putative factors both in methicillin-sensitive S. aureus (MSSA) and MRSA infection in mice. Effects were analyzed in terms of animals secretion level for inflammatory markers such as IL-6, IL-8, IL-1b, IL-10, and keratynocyte chemoattractant (KC), and more detailed study was carried out to assay the level of IL-6 and macrophage inflammatory protein (MIP-2, CXCL2) at delayed stages of infection (72 h post-infection). PRP-1 and analogues stimulates higher production of both pro- and anti-inflammatory cytokines such as IL-6, IL-10, IL-1b, TNFα and KC at early stage of infection, and for more delayed period (72 h post-infection) had significant impact on the plasma levels of the main markers of inflammation – IL-6 and MIP-2. Received data provide further insight into the intricate cytokine activity involved in the regulation of inflammatory responses and the control of bacterial infections and allow concluding that PRP-1 and its analogues are potential immunostimulatory agents for MRSA-induced infection prophylaxis and treatment in vivo.

SW04.S16–4
Binding polyspecificity and catalytic polyreactivity of human milk immunoglobulins is due to various combinations of antigen-binding sites
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Natural immunoglobulins are considered as monospecific molecules that have stable structures and two or more identical antigen-binding sites. We have shown that IgG and sIgA of human milk eluted from affinity sorbents under the conditions destroying strong complexes demonstrate high catalytic activities.
in hydrolysis of ATP, DNA, oligosaccharides and phosphorylation of proteins, lipids and oligosaccharides.

In preparations of human milk IgG and sIgA we have found chimeric λ2-IgGs and λ2-sIgAs, which contain both types of immunoglobulin light chains simultaneously. Chimeric λ2-IgGs were presented by all four subclasses (IgG1-IgG4). Moreover, adding of reduced glutathione and milk plasma containing no antibodies to IgG or sIgA fractions with different affinity for DNA-cellulose leads to antibodies transition from one fraction to the other. We explain this phenomenon by the possibility of HL-fragments exchange between different IgG, IgA and sIgA molecules, but not the exchange of only light or heavy chains. Some chimeric molecules of sIgA may contain from two up to four HL-fragments to various antigens high affinity interacting with different sorbents and catalyzing various chemical reactions.

Our data essentially expand the ideas explaining the phenomenon of polyspecificity and cross-reactivity of human milk antibodies. Further investigations will be focused on allocation of human milk factor that provides exchange of immunoglobulin structural components. This could lead to a method of producing bispecific antibodies.

**SW04.S16–5**

**Immunogenicity and protective efficacy of prime-boost regimens with recombinant multivalent BCG and modified vaccinia virus Ankara expressing tuberculosis antigens providing protection against Mycobacterium tuberculosis**

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Tuberculosis caused by infection with Mycobacterium tuberculosis remains one of the most important emergent infectious diseases of man. The current vaccine, M. bovis bacillus Calmette-Gue'rín (BCG) demonstrates variable efficacy in humans, and so an urgent need exists for a new regimen to replace or supplement BCG. In the present study, we established two promising new vaccine candidates are the multivalent recombinant BCG (multi-rBCG), which has been developed to replace the current tice BCG vaccine strain, and the modified vaccinia virus Ankara (MVA) expressing M. tuberculosis antigens (multi-rTB MVA), which is a leading candidate vaccine designed to boost the protective efficacy of BCG. We examined the effect of multi-rTB MVA boosting on the protection afforded at 4 weeks post-challenge by tice BCG and multi-rBCG by using bacterial CFU as the efficacy readout. Both of the multi-rTB MVA-immunized and multi-rBCG-immunized animals were significantly better protected against aerosol challenge with M. tuberculosis than animals immunized with the parental strain of BCG. To identify the immunological surrogate markers for the development of a protective immune response against M. tuberculosis challenge, we analyzed immune response after priming or prime-boosting by using cytoplasmic cytokine detecting and assays for cytokine secretion in mice, guinea pigs, and non-human primates, respectively. We show that the numbers of systemic multifunctional cytokine-producing cells do correlate with protection against aerosol challenge in animals. multi-rTB MVA vaccination of intranasal challenged mice and guinea pigs induced 3 logs of protection in the lung with similar kinetics as those displayed in M. tuberculosis aerosol infection models. Boosting with both of the multi-rTB MVA and multi-rBCG, but not priming with tice BCG, greatly increased the tuberculosis antigens-specific CD4+ and CD8+ T-cell response, suggesting that the mechanism of protection may differ from that against BCG. Thus, multi-rTB MVA and multi-rBCG vaccination capable of inducing efficient cell-mediated immunity might be used as an effective vaccine for tuberculosis.

**SW04.S16–6**

**Immunocytochemistry approach in diagnosis of immunodeficiency in sportsmen**

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**Research aim:** Investigate the cell immunity markers and activity of succinate dehydrogenase (SQR) in different lymphocyte populations in young sportsmen undergoing basic training process.

This research was based on the sample of 100 children from 9 till 18 years old who regularly attended youth swimming sport school. Investigation of subjects included basic clinical examination and immunophenotyping of lymphocytes according to advanced protocol with identifying the amount of cells in minor populations and assessment of SQR activity in typed populations of lymphocytes. All children were divided into two age groups: 9–12 years old (35 children), 12–18 years old (65 children). Children in each age group were further subdivided into groups according to the results of clinical examination: healthy subjects, subject with identified nidus of chronic infection and subject with presence of cardio-vascular malfunctions. Based on this classification, there appeared two subgroups in the younger age group: healthy subjects (25) and subject with present nidus of chronic infection (10) and 3 subgroups in older age group: healthy subjects (35), subject with present nidus of chronic infection (15) and subjects with cardiovascular malfunctions (CVM) (15).

The research showed immunological aberrations in young athletes of both age groups with more significant deviations in older age group. The examination of children from younger age group with chronic infection detected the decrease in the amounts of T-cytotoxic lymphocytes and normal activity of SQR in them and increased activity of SQR in activated T-lymphocytes (HLA DR). Sportsmen from older age group in all three subgroups appeared to have constant increase in NK cells with ongoing increase in activity of SQR in children with chronic diseases and cardio-vascular malfunctions. Additionally, it was noticed that older children with chronic inflammation process have more T-cytotoxic and activated T-lymphocytes (HLA DR) with increased activity of SQR in them. The analysis of minor populations of lymphocytes in younger children detected no differences between subgroups in desired parameters. However, the research showed significant increase in SQR activity in activated T helpers and TH-2-lymphocytes in older children with chronic inflammation process and B1-lymphocytes in older children with CVM.
SW04.16–7
Blood complement proteomics of mother – umbilical cord – newborn triad. Lack of complement C1 inhibitor at newborns of risk group correlates with infection development
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The work is aimed to the immunochemical study of diagnostic potential of level and functional activity of complement system C1 inhibitor in newborn blood and in their mothers from a risk group of infection development. We have studied the blood samples from newborns and their mothers from a group at risk of developing micoplasma and ureaplasma infections. A total 105 blood samples have been studied: from umbilical cord; from corresponding newborns at the 3–5th day of life; and from their mothers while giving birth. The blood of 10 healthy newborns, their mothers and the umbilical cord blood were used as a control group for comparison. Mothers of newborns from the risk group have the following PCR-positive infections: micoplasma and ureaplasma – 100%; cytomegalovirus – 32%; herpes – 32%; chlamydia – 16%. It is found that there is reliable increased activity of the control newborn C1 inhibitor level (p < 0.009) compared to the umbilical cord blood. There are no valuable differences between C1 level at risk newborns and their umbilical cord blood. Umbilical cord C1 inhibitor minimal level and activity values are: 142.7 ± 13.47 mcg/ml and 130.3 ± 10.1 mcg/ml. There is valuable increase of C1 inhibitor activity (p < 0.009) in the newborns’ blood: 178.1 ± 13.2 mcg/ml, C1 level being slightly increased 159.9 ± 14.8 mcg/ml. It could result in weakening the activation of classical and alternative pathways observed earlier. C1 activity at newborns from infected mothers is much higher compared to umbilical cord: reliability at newborns is high by one other of magnitude. C1 inhibitor activity in newborns from infected mothers is remarkably higher, and equaled to 298.79 ± 33.56 mcg/ml (p < 0.003). C1 inhibitor level is also increased in newborns from infected mothers: 216.5 ± 32.2 mcg/ml. These mothers are characterized with very high values of C1 inhibitor level and functional activity: 602.6 ± 54.78 mcg/ml and 432.8 ± 49.8 mcg/ml, correspondingly. Thus, minimal C1 inhibitor level and functional activity are characteristic for the umbilical cord blood; sharp increase of C1 functional activity is characteristic for newborns. Mothers infected before labor are characterized with an increase of blood C1 functional activity in their newborns.

SW04.16–8
Nanoprobes on the basis of fluorescent semiconductor nanocrystals for bioassays and biosensing
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Some features of interaction between 2′–5′ and 3′–5′ oligoadenylates with proteins detected by MALDI-TOF mass spectrometry
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Previously during clinical tests it was shown that artificially synthesized ‘core’ 2′–5′ oligonucleotides which contain three or more elementary links - adenine or its derivatives, can be used as immunomodulators, antivirus and anticancer drugs [1]. The mechanism of oligoadenylate’s unique biological activity is directly related to their ability to interact with target proteins [2]. From the other hand it’s well-known that the main role in antiviral cell defense belongs to s-interferon. So it’s very important to find method that allows us not only detect interaction between ‘core’ 2′–5′A3, its modified analogue with proteins (especially with interferon) but also reveal some its features. For solving these tasks we used MALDI-TOF mass spectrometry. Analysis of oligoadenylates, proteins and oligoadenylates – proteins system by mass-spectrometry showed that interferon can bind up to 5 molecules of synthetic oligoadenylates 2′–5′A3. The similar situation was observed for natural oligomer 3′–5′A5. But in a case of 2′–5′A3-epo it was shown that interferon are capable of binding only up to 3 2′–5′A3-epo molecules. Possible mechanisms of interaction of oligoadenylates with interferon are discussed. At the same time none of these oligoadenylates showed the ability bind to insulin. So all investigated oligoadenylates, regardless of their length and structural features, are show tendency to valuable tools for diagnosis, imaging, optical tracking and sensing. Semiconductor nanocrystals are very promising alternative for organic dyes due to their excellent photostability, high quantum yield and narrow emission peak that depends only on QD size. Multicolor and possibility of excitation of different colors QDs incorporated in bead by single irradiation source, allows create multiplexed diagnostic systems. The use of so-called ‘smart’ polymers permits to create sensor systems for defining parameters of medium in local volumes of nanometric range. We considered two classes of hybrid nanosystems: (i) nanoprobes for imaging, identification and diagnosis, and (ii) nanosensors for measuring of temperature, pH and the concentration of copper ions in nanovolumes.

Common strategy for diagnostics with QDs utilizes the specificity of monoclonal antibodies (mAbs) for targeting. Here, we engineered ultrasmall nanoprobes through oriented conjugation of QDs with 13-kDa single-domain antibodies (sdAbs) derived from llama IgG. This approach allowed us to develop sdAbs-QD nanoprobes comprising sdAbs coupled with a QD in a highly oriented manner. The conjugates sdAbs-QD demonstrated excellent specificity in flow cytometry analysis, and for immunohistochemical labeling of biopsy samples.

Composite polymer particles sensitive to pH, ion concentration and temperature of physiological range were prepared by the layer-by-layer deposition technique. Polyacrolein particles are used as cores for deposition of hydrophilic QDs/polyelectrolyte multilayers. The fluorescence of such polymer particles with bovine serum album in outer layer is sensitive to copper (II) ion while the fluorescence of these particles is practically insensitive to other divalent cations. Use of the polymer particles consisting of a poly(N-vinylcaprolactam) polymer shell doped with QDs permits to create themosensitive microparticles. These two classes of nanobiohybrid materials represent a new generation of probes for applications in high-throughput diagnostic platforms.
multiple interactions multiple interactions with α-interferon and don’t interact with insulin. Therefore α-interferon is one of the target proteins for 2′–5′, 3′–5′ oligoadenylates and its derivatives.

From the data obtained we can conclude that using of mass spectrometry method in the protein-oligonucleotides interaction studies give us the possibility to clarify the mechanism of oligoadenylate biological activity and find proteins which are involved in it. Also these data give the background for discovering new antiviral and anti-inflammatory drugs based on oligoadenylates and its analogues.

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4. A. A. Bogdanov1, P. V. Sergiev1 and O. A. Dontsova1
5. Successful introduction of a new antibiotic in practice it is neces-

SW04.S16–10
Screening for small molecules that disrupt epigenetic silencing in mammalian cells
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Epigenetic silencing is essential for modulating gene expression patterns in developing organisms and has a very big impact on the genome integrity. Cancer, as well as many other diseases, has been linked to the changes in transcription of regulatory genes due to the aberrant epigenetic silencing. Therefore the epigenetic regulators, enzymes including histone deacetylases, methyltransferases, demethylases, DNA methyltransferases, and other enzymes contributing to chromatin structure modulation, are targets for anti-cancer drugs. Here we employ a screening assay in mouse erythroid leukemia (MEL) cells that harbor a silenced green fluorescent protein (gGFP) reporter transgene in order to isolate new components of heterochromatic silencing and identify compounds that have potential to de-repress tumor suppressor genes in cancer cells. This assay allowed us to rank known inhibitors of epigenetic pathways with relation to their anti-silencing properties. Furthermore we have tested a library of known kinase inhibitors and identified a number of inhibitors that release silencing. Thus, particular kinases may have previously undocumented roles in chromatin modulation. We next assayed those compounds with strong anti-silencing properties for their ability to de-repress silenced tumor suppressor genes in cancer cell lines, and to affect proliferation of these cell lines.

SW04.S16–11
New system for high-throughput search of the bacterial translation inhibitors
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Each year number of pathogenic bacteria with resistance to modern antibiotics increases. The finding of new antimicrobial compounds, usually is made by means of high-throughput screening of synthetic chemicals or products of microbial metabolism. For successful introduction of a new antibiotic in practice it is neces-

SW04 Molecular Mechanisms of Disease

SW04.S16–12
The influence of tobacco smoking on glutathione status in the blood analyzed by capillary electrophoresis
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Cigarette smoking leads to the uptake of a multitude of reactive chemicals including many electrophiles and may give rise to oxidative stress. Glutathione is one of the most significant components of collective antioxidant defense [1] and determination of reduced (GSH) and oxidized glutathione (GSSG) in the blood is an useful indicator of disease risk in humans [2].

The purpose of the present work was to compare the glutathione concentration in the blood of smoking and non-smoking women, therefore the rapid and sensitive capillary electrophoresis method for measuring both, GSH and GSSG in the blood was used.

In the group of smoking women was observed the lower concentration of reduced glutathione (445.11 ± 67.46 μM) compared to the non-smoking group (595.8 ± 158.63 μM). Simultaneously, the oxidized form of glutathione was measured, what allowed for more accurate interpretation of the glutathione status. Higher GSSG concentration in the blood of women of smoking group (335.53 ± 75.79 μM), than in nonsmoking group (272.73 ± 68.58 μM) was observed.

Elevated concentration of GSSG could be the result of the production of free radicals and oxidizing agents when smoking, which may convert GSH into its oxidized form.

References
Annexin V: the old molecule with a new application

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Placenta is an organ with functions such as synthesis of hormones, creation of favorable microenvironment for fetus development, protection of mother organism from harmful factors participating in the process of fetus growth. However, besides such functions placenta synthesizes also hormones and proteins which are directly involved in the processes of embryogenesis. Since such factors are revealed also in carcinogenesis, placenta can be used for searching of markers of carcinogenesis. As placenta is a rich source of annexin V which function is not completely elucidated, we tried to test its role as a marker of carcinogenesis. For this purpose antibodies to annexin V sensitized on polystyrene latex particles were applied for detection of cancer. Our investigations demonstrated the existence of annexin V in sera of pregnant women and its concentration is lowered during the pregnancy. It was shown also its existence in sera of patients with multiple myeloma, breast, prostate and ovary cancer. In contrast to some markers of carcinogenesis which are specific for only a definite type of cancer (chorionic gonadotropin, CA-19.9, CA-125 etc), annexin V can serve as nonspecific marker for different tumors and possibly can be applied in screening diagnosis of cancer.

Application of CdSe quantum dots for simultaneous determination of two antigens in homogeneous immunoassay

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Quantum dots (QD) are highly luminescent, photostable fluorophores that are considered as very prospective semiconducting nanoparticles for labeling and sensing of biomolecules and cells. Fluorescence emission wavelength can be tuned by modifying the size of QD particles and the type of capping molecules. Various lots of CdTe quantum dots with different emission wavelengths were applied for simultaneous determination of two antigens by the method of fluorescence resonance energy transfer (FRET). This approach was applied for simultaneous determination of C-reactive protein (CRP) and ferritin. One lot of nanoparticles with emission wavelength at 520 nm was sensitized by antibodies to C-reactive protein and the other lot of nanoparticles with emission wavelength at 540 nm was sensitized by antibodies to ferritin and finally the last lot of nanoparticles with emission wavelength at 580 nm was sensitized by antibodies to human IgG F(ab) fragments. The absence of any antigen will bring to FRET and as a result fluorescence at 520 and 540 nm will be decreased with consequent increasing of emission at 580 nm. The presence of both antigens in sample will conserve fluorescence properties of all lots of QD. Presence of only one of antigens will bring to decrease of emission intensity of one of the lot whereas the other lot will decrease its fluorescence as a result of FRET. Effects of various parameters such as concentration of nanoparticles, sensitized antibodies, methods of immobilization on sensitivity and specificity of assay were tested. The possibilities of this approach for simultaneous determination of multiple antigens are discussed.

Thiol-disulfide system as universal biomarker for personalized therapy of infectious diseases and cancer

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The purpose of this study was the search of new biomarkers for personalized therapy of cancer and infectious diseases. Thiol-disulfide system is selected as a target for drug screening as the most important biochemical system that characterizes the state of the antioxidant defense and redox processes. Currently, the level of plasma or serum thiols, including protein and non-protein thiols (SH) and thiol-disulfide (SH/SS) ratio of blood are considered as indicators of the state of immune competence and nonspecific resistance. The study involved more than 1200 patients with chronic hepatitis C (HCV), genitl herpes, human papillomavirus infection, chlamydiosis, chronic bronchitis, endometriosis, non-small cell lung cancer (NSCLC), metastatic kidney and prostate cancer. An original method for screening drug preparations was proposed (RU 2 150 700; US 6 627 452; EP 1 182 455), where whole blood with anticoagulant (EDTA) was incubated in thermostat (37°C) in the presence of drugs for 1 h, the control samples were incubated with saline. Determination of SH-and SS-groups in hemolysate was done by spectrophotometry. In processing the data performance SH-groups and SH/SS ratio in the control and experimental samples were compared. It was found that all medications, including antibiotics, cytostatics, hormonal, antiviral drugs and immune preparations have a stimulating or depressing effect on blood thiol-disulfide system, by which one can predict the efficacy and safety of therapy, and to individualize treatment. In controlled studies a 3-fold increase in the incidence of virological response of personalized monotherapy with interferon (IFN) of HCV patients was shown, and the incidence of adverse effects was decreased by six times compared with standard IFN therapy. Prognostic significance of SH/SS-test for the treatment of HCV patients was 89.8%. Controlled studies also demonstrated a 2-fold increase in the efficiency of personalized hormonal therapy of endometriosis compared with standard and a 2-fold increase in the frequency of partial remission rate of NSCLC after personalized treatment by cytostatics compared with standard chemotherapy. At exacerbations of chronic bronchitis the incidence of unsatisfactory clinical results or recurrence was observed in three times less, unsatisfactory bacteriological result - four times less and incidence of side effects – 2 times less. Thus, thiol-disulfide system can be regarded as a universal biomarker for personalized treatment of infectious diseases and cancer. The proposed method for screening drugs using SH/SS-test can be used to personalize therapy by antibiotic, hormonal, antiviral, cytostatic and immune preparations of infectious diseases and cancer to improve efficiency and overcome resistance to drugs, finding the best drugs, their doses and combinations, reducing the frequency of side effects and complications.
SW04.S16–16
Development of a Multiplex-PCR assay for the simultaneous detection and identification of eight Lactobacillus strains in clinical samples
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Introduction: The healthy human vagina is dominated by Lactobacillus, playing an important role in protecting the woman from genital infections. Distinct species of Lactobacillus have been identified as vaginal commensal. However, some dissimilarity in prevalence results can be recognized in published work. Identification methods based on in vitro culture techniques and biochemical methods may contribute to this non-consensual data. Such methods suffer from several drawbacks. Consequently, rapid and accurate genomic molecular methods are required in order to identify properly Lactobacillus species in vaginal samples.

Methods: A Multiplex-PCR procedure was developed for the simultaneous detection and identification of eight Lactobacillus strains, L. jensenii, L. acidophilus, L. crispatus, L. iners, L. gasseri, L. plantarum, L. rhamnosus and L. reuteri from ninety clinical samples (20 swabs, 30 vaginal fluid samples and 40 isolates). The reaction mixture contains eight species-specific primer pairs and reaction conditions were optimized in order to achieve equal amounts of amplification products. The performance and specificity of procedure was tested in 22 collection strains.

Results and Discussion: The established assay allowed the identification of eight Lactobacillus species in different clinical vaginal samples. The prevalent species on analyzed samples were L. crispatus, L. iners and L. gasseri. These results are in accordance with other reports that also refer these species as the prevalent ones. A detection level of approximately 50 bacterial cells per reaction of the study organisms was achieved and no unspecific products were amplified on PCR assay.

Conclusions: The developed multiplex-PCR was shown to be able to identify eight Lactobacillus species in vaginal samples with a high specificity and reproducibility. This procedure allows to study probiotic flora in vaginal samples, preventing culture-dependent procedures that exhibit several limitations. This procedure is proposed as a simple, fast and reliable method for identification of common Lactobacillus from vaginal clinical samples.

SW04.S16–17
Identification of inherited disorders by tandem mass spectrometry analysis of dried blood spots
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The identification of metabolic disorders, mainly inherited, is an important goal of modern science and medicine. High-tech methods of analysis with using modern equipment are used for this purpose in Europe and the United States. Usually newborn screening, a blood test of newborns for 40 different diseases, is carried out by mass spectrometry and selective genotyping of individual genes polymorphisms. In Russia since the early 90’s the screening tests for phenylketonuria and congenital thyroid deficiency are throughout obligatory. Since 2006 adrenogenital syndrome, galactosemia and mucoviscidosis were added in newborn screening. Expanding the list of identified disease is impossible without the introduction of innovative technologies. Unfortunately, to date, a range of inborn errors of metabolism remains outside the neonatal screening used in Russia. These diseases often follow not immediately. With age the disorders declare itself and delays in mental and physical development are evident. For a long time doctors cannot diagnose diseases correctly due to lack of analytical methods. The using of mass-spectrometry for identification of amino acids and acylcarnitines metabolic disorders in children 1 year age and older provides a solution to this problem.

The purpose of this study is to determine the level of normal amino acids and acylcarnitines in blood of children 1 year and older, living in the Novosibirsk region.

A total amount of 321 patients with a history of violations of physical and mental development were participated in the study. Age of patients was 29 days – 7 years old, the average age – 3 years, among them male – 189 (59%) patients, women – 131 (41%) patients. Blood of patients was placed on a special paper and dried.

Dry blood spots were used to determine the concentration of amino acids, organic acids and acylcarnitines by mass spectrometry instrument Agilent 6410 QQQ (Agilent Technologies).

As a result of statistical data were obtained: mean – X, SD – s, 95% confidence interval – X ± 2s for 12 amino acids (Ala, Arg, Asp, Cit, Glu, Gly, Met, Orn, Phe, Tyr, Val, Xle) and 12 major acylcarnitines (C0, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16, C18).

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SW04.S16–18
Synthesis of triazole-linked oligonucleotides, which are usable as PCR primers
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Oligonucleotide (ON) analogs with triazole internucleotide linkages have shown great promise in the field of antisense technology and as DNA hybridization probes. A series of ON analogs bearing the triazole modification were obtained by automated solid-phase synthesis with a modified dinucleoside phosphoramidite block. The dinucleoside block was obtained by click- ligation of 3′-O-azidomethyl and 5′-deoxy-5′-ethynyl nucleoside derivatives. The properties of the triazole-linked ONs are described. The analogs demonstrated DNA binding affinities similar to those of unmodified oligonucleotides. The modification was shown to protect oligonucleotides from nuclease hydrolysis. The modified oligonucleotides were tested as PCR primers. Modifications remote from the 3′-terminus were tolerated by Taq and Pfu polymerases. The sensitivity of Taq to primer modification was evaluated rigorously by qPCR. Modifications at up to eight nucleotides from the 3′ end resulted in a substantial decrease in the product accumulation rate. Thus, the new triazole-linked ONs are generally usable as PCR primers, but the dependency of PCR efficiency on the modification position should be taken into account. Modified PCR primers may be useful for SNP-based allele detection should they demonstrate an enhanced sensitivity to mismatches.
**SW04.S16–19**

Quantification and validation of a liquid chromatography-tandem mass spectrometry assay for serum busulfan levels

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Busulfan is a cellcycle cycle non-specific alkylating antineoplastic agent, in the class of alkyl sulfonates. Currently, its main uses are in bone marrow transplantation especially in chronic myelogenous leukemia (CML), where it is used as an alkylated agent. A sensitive and high-throughput LC-MS/MS method has been developed and validated for the determination of busulfan in human plasma with d8 busulfan as internal standard. Chromatographic separation was achieved in 2.0 min on Acquity BEH C18 column using methanol/acetonitrile (90:10)–50 mM ammonium formate% 0.1 Formic acid as mobile phase. Mass detection was achieved by ES/MS/MS in positive ion mode, monitoring at m/z 264.10 and 151.10, 272.10 for busulfan, and IS, respectively. The calibration curves were linear from 10.00 to 1000.0 ng/ml for busulfan. The intra-day and inter-day accuracy and precision were within the acceptable limits of 10.00–1000.0 ng/ml for busulfan concentrations. The assay is rapid and sensitive for measuring serum busulfan and it is suitable for clinical application.

**SW04.S16–20**

Effect of DNA on the sensitivity, specificity and efficiency of three multiplex real-time PCR in different types of clinical specimens for rapid detection of extrapulmonary tuberculosis and focal complications of brucellosis

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Tuberculosis and brucellosis are worldwide diseases. The differential diagnosis between extrapulmonary tuberculosis and forms of focal brucellosis based on clinical, laboratory, imaging and even pathologic features is difficult. The real time PCR has proven faster and more sensitive than conventional methods in both diseases. The quality and amount of DNA from clinical samples can affect amplification efficiency. Previous studies on the choice of targets showed better results for gene combinations using bscp31 or IS711 targets for Brucella and sens3-regx3 or IS6110 for M. tuberculosis complex (MTC). Our aim was to analyze the sensitivity, specificity and efficacy of three multiplex real-time PCR in different types of clinical samples for potential use in the simultaneous detection of Brucella and MTC. A total of 86 clinical specimens were processed and analyzed by multiplex real-time PCR. The gene combinations used for the identification of Brucella and MTC were sens3-regx3+bscp31, sens3-regx3+IS711 and IS6110+IS711. DNA from different clinical samples was extracted using different methods. Melting curve analysis correctly identified 96.51% of patients with brucellosis and tuberculosi, showing negative results in all the controls and for the three multiplex assays. The amplification efficiency was not affected by the DNA quality of the different types of samples, though it was affected by the amount of total DNA. Thus, the amplification sensitivity decreased with increasing amounts of DNA in the reaction. In conclusion, the presence of large amounts of human DNA in PCR reactions decreases the sensitivity and detection limits of the technique. This factor appears to be more important than the quality of the DNA and must be considered for the design of PCR assays. The three multiplex PCR assays are highly reproducible and efficient, and may be a promising and practical approach for the rapid differential diagnosis of extrapulmonary tuberculosis and brucellosis.

**SW04.S16–21**

Search of differentially expressed genes in peripheral blood leukocytes for diagnostics of atherosclerosis

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Preventive treatment of atherosclerosis is one of the most important goals of modern medicine. Despite many years of research, there are no techniques of early diagnostics of this disease. A substantial part of patients develop atherosclerosis even with low assessed risk. A DNA microarray analysis offers a way of early atherosclerosis diagnostics: it revealed genes, which expression in peripheral blood leukocytes correlates with the extent of coronary artery disease. A DNA microarray based non-invasive blood test that permits early estimation of cardiovascular events risk would have great medical implication.

In the present study we used DNA microarray technology to obtain gene expression profiles of peripheral blood leukocytes of 32 patients with angiographically confirmed carotid artery stenosis and 14 individuals who had no clinical symptoms of atherosclerosis.

For gene expression profiling were used Atlas™ cDNA expression arrays (BD Biosciences Clontech): Cardiovascular, Human M7740 and Human 1.2 II. After normalization of signal intensity differentially expressed genes were selected. Obtained data were confirmed by quantitative real-time PCR, using real-time detection thermal cycler DTprime (DNA technology). PCR data were hierarchically clustered.

Cluster analysis of PCR data shows that chosen set of genes allows to distinguish between most of individuals without clinical symptoms of atherosclerosis and patients with angiographically confirmed carotid artery stenosis. About third part of subjects without clinical symptoms of atherosclerosis have gene expression profiles, which are similar to gene expression profiles of patients. Among the differentially expressed genes were found transcription factors, regulators of cell proliferation and differentiation, receptor coding genes, tumor suppressors, apoptosis regulators and early response genes.

**SW04.S16–22**

Method characteristics of 17 OH progesteron by tandem mass spectrometry

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Objective: Steroid measurements play an important role for diagnosing endocrine disorders. Prior to the introduction of mass spectrometry (MS) in clinical laboratories, the main techniques for measurement of steroids were immunoassays (IA). Introduction of IA in clinical laboratory testing was a significant milestone that transformed practices in endocrinology (1). These methods have proven to be cost-effective and sensitive, but they
have limitations with respect to specificity (2). The aim of this study was to evaluate the method performance characteristics of liquid chromatography-mass spectrometry (LC-MS/MS) assay for determining serum 17-OH progesterone.

**Material and Methods:** After a liquid-liquid extraction (LLE) with diethylether/ethylacetate, organic phase was dried under nitrogen gas. Dried extract was dissolved in 50% methanol/water. The analysis of serum 17-OH progesterone was carried out on ABSCIEX API 3200 LC-MS/MS system. Method performance characteristics were evaluated with EP Evaluator Release 8 (DRG Roads).

**Results:** Imprecision was performed for three different 17-OH progesterone concentrations (0.8, 6, 12 ng/ml). With-in run, with-in day, and between-run coefficient of variation (CV) values were 7.81, 5.65, 3.72%; 2.10, 3.67, 1.50% and 3.70, 5.37, 3.13%, respectively. Linearity and accuracy were calculated against a calibration curve. Calibration was linear up to 25 ng/ml. The calculated bias was 2.2–2.8%. The recovery was between 98.4 and 99.2%. Limit of detection and limit of quantification were 0.1 and 0.3 ng/ml, respectively.

**Conclusion:** According to these results, determination of serum 17-OH progesterone by LC-MS/MS offers more precision compared to ELISAs.

**SW04.S16–23**

**Selection of DNA aptamers specific to Shiga toxin 1 of *Escherichia coli* O157:H7 for the development of diagnostic assays**

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Shiga toxins (Stx1 and Stx2) are recognized virulence factors of *E. coli* O157:H7, invoking diarrhea, hemorrhagic colitis, and a life-threatening hemolytic uremic syndrome (HUS). Conventional diagnostic procedures for Stx detection are based on immunological methods, PCR techniques and the Vero cell cytotoxicity assay. Each of these methods has certain drawbacks; the most significant among them is limited sensitivity of Stx diagnostic tests. The sensitivity of Stx detection can be enhanced by the employment of immuno-PCR assay based on aptamers - short fragments of DNA, which are capable of direct and specific binding to the target. The purpose of the present study was to obtain specific DNA aptamers for development of immuno-PCR aptamer-based assay for rapid and efficient detection of Stx1.

In order to facilitate specific aptamer selection we have constructed chimeric recombinant protein comprised of glutathione-S-transferase, peptide, being a specific substrate of *B. anthracis* lethal factor protease and B-subunit of Shiga toxin 1. This protein was immobilized on magnetic beads carrying glutathione and incubated for 1 h at 37°C with a random oligonucleotide library (81 base in length) with constant flanking regions. Next to several rounds of washing to remove unbound oligonucleotides, B-subunit of Stx1 with specifically bound aptamers was cleaved off by addition *B. anthracis* lethal factor metallo-protease. Thus, we deleted non-specific DNA sequences bound to the matrix on which the target protein has been immobilized. Specific aptamers were amplified by PCR with primers to their constant regions. One of those primers was phosphorylated at 5’end, and the other contained a fluorescent label (FAM). In order to obtain single-stranded DNA for the next round of aptamers selection, the PCR product was treated with recombinant 5’- exonuclease of lambda phage. The destruction of non-fluorescent phosphorylated DNA chain was controlled by 10% PAGE. After the five rounds of selection, the resulted sequences were cloned into the standard plasmid vector (pBluescript II SK-).

A panel of DNA aptamers with high affinity and specificity to Shiga toxin 1 was obtained using the procedure described above. Interaction of aptamers with Stx1 was characterized by high binding constants (up to 10^9 M⁻¹). The selected panel of aptamers can be used as a basis for elaboration of highly specific and sensitive diagnostic tools.

**SW04.S16–24**

**ADMA and total oxidant status in saliva**

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Asymmetric dimethylarginine (ADMA) regulates nitric oxide generation in numerous disease by inhibiting nitric oxide synthase (NOS). Increased oxidant activity is implemented for the development of periodontal disease and increased caries risk. The aim of this study is to evaluate ADMA levels and total oxidant status (TOS) in saliva.

**Methods:** ADMA levels were measured by HPLC with fluorescence detection in 80 saliva samples. The TOS levels of the saliva were determined using a novel automated measurement colorimetric method. Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complexes into ferric ions.

**Results:** Twenty-one patients were determined as periodontitis. There were no significant difference between the ADMA, arginin levels and TOS activities between the patients and control groups. TOS activities showed a negative correlation with saliva arginin/ADMA levels (r: –0.24; p: 0.034).

**Conclusion:** Increased TOS activity correlated with increased turnover of arginine to ADMA. Therefore we can say ADMA increase oxidant activity possibly by inhibiting NOS and decreasing NO bioavailability.

**SW04.S16–25**

**Developing a Mycobacterium smegmatis based test-system for screening mycobacterial protein kinase PknB inhibitors – potential next generation anti-tuberculosis drugs**

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*Mycobacterium tuberculosis* (*Mtb*), the main infective agent causing tuberculosis (TB), has infected over 1/3 of the World population, causing over 9 million newly registered cases of TB and 1.4 million deaths in 2011. The long-term use of the current drug regimen has resulted in the emergence of *Mtb* strains with multidrug-resistance (MDR-TB), and extensive drug-resistance (XDR-TB). Therefore, there is a need in search for next generation anti-TB drugs with a novel targeted mechanism of action. We consider eukaryotic-type serine/threonine protein kinases (ESTPKs) as effective biotargets for next generation anti-TB therapy [1]. *Mtb* genome contains 11 ESTPK genes, including PknB, which is essential for cell growth and division.
We've developed a test-system for screening protein kinase inhibitors based on *M. smegmatis* ms2 155 strain, carrying 13 ESTPK genes, highly homologous to *Mtb* ESTPKs. The key element of this system is aminoglycoside-O-phosphotransferase VIII (APHVIII) from *Streptomyces rimosus*, which makes cells resistant to aminoglycoside antibiotics [2]. APHVIII can be phosphorylated by ESTPKs on S-146 site, leading to increased kanamycin resistance. The addition of the ESTPK inhibitor prevents phosphorylation and makes the cells more sensitive to kanamycin.

The APHVIII gene was cloned in a modified pMIND shuttle vector under Tet-inducible promoter and electroporated in *M. smegmatis*. Transformant cells were resistant to 250 µg/ml kanamycin, and the resistance decreased when standard ESTPK inhibitors of indolylmaleimides class were applied. In order to increase specificity of phosphorylation by PknB, eight modifications of APHVIII with mutant S-146 site were made by site directed mutagenesis and expressed in *M. smegmatis*.

The *M. smegmatis* APHVIII+ test-system has been validated with standard PknB inhibitors, and was used to screen PknB inhibitors among compounds of azolotetrazines class. The most active compound, Tritrazine-N, now undergoes preclinical tests as a next generation anti-TB drug [3].

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References

SW04.S16-26

Study of pentamethinium fluorescent probes, indolium versus benzothiazolium probes and effect of gama substitution

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Labeling of mitochondria for fluorescence microscopy is generally achieved using transiently expressed mitochondrial protein markers or dyes specifically accumulating in this organelle. We demonstrate a series of novel fluorescent dyes from γ-aryl substituted pentamethine with side indolium units possessing excellent photo-stability and fluorescence properties. They localize in mitochondria of various cell lines with unique selectivity and are detectable in nanomolar concentrations. An influence of type of side heteroaromat on photo-stability is presented. Moreover, indolium unit is responsible for new properties in comparison with benzothiazolium units.

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SW04.S16-27

Diagnostic value of the minor lymphocytes subsets succinate dehydrogenase activity in children with autoimmune disease

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Introduction: There is no doubt nowadays, that any pathologic process leads to the cells energy metabolism disbalance, which is based on the functional disorders of mitochondria. The succinate dehydrogenase (SDH) is both respiratory chain and Krebs cycle enzyme) activity measurement is a widespread method of the cells bioenergetic condition evaluation. The measurement of the SDH activity in the minor peripheral lymphocytes subsets (immunocytochemical analysis) helps in the estimation of the cells immunity parts functioning and opens up new diagnostic possibility in patients with immune mediated diseases.

Patients and methods: We studied 44 children with Crohn’s disease (CD) and 26 patients with ulcerative colitis (UC) at the age from 6 to 18 years, who received infliximab for a long period. Immunocytochemical analysis was carried out by flow cytometry (flow cytometer Beckman Coulter FC500).

Results: We showed that children with CD and UC had statistically significant decrease of the B-cells number and their SDH activity and aggravated with the disease duration increase. BI-lymphocytes population was less in patients with UC and CD compared to healthy children – median 12.5 ± 0.3% versus 30.9 ± 2.6% of B-cells, on the contrary SDH activity was higher in patients with UC and CD (153.6 ± 6.4 relative unit (r.u.) than in healthy children (108.3 ± 3.9 r.u.). Gradual intense growth of the SDH activity and BI-cells number was observed in patients with nonsustained effect of infliximab. Initial number of NK-cells was half the size of this population in patient with UC and CD (6.1 ± 1.1%) compared to healthy children (15.5 ± 1.2%), NK cells SDH activity was increased compared to the healthy persons (192.5 ± 8.1 r.u. versus 161.0 ± 3.8 r.u.). T-cells immunity analysis showed that activated T-helpers predominated over regulatory T-cells population in children with nonsustained effect of infliximab. Activated TH-lymphocytes SDH activity was higher in patient with CD and UC than in healthy children.

Conclusion: Thus, our data suggest that the assessment of minor lymphocytes subsets SDH activity has diagnostic value and can be recommended as adjuvant criterion in the follow-up of children with inflammatory bowel disease, especially receiving anti-cytokine therapy.

SW04.S16-28

Cytological Investigation of saliva samples of disabled children

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Children that fall behind their peers in mental, physical, social and medical aspects are defined as ‘children with disabilities’. Caries incidence varies according to the type of disability among disabled children. Moreover children with disabilities are often reported to be associated with poor oral hygiene and periodontal problems. Saliva is of great importance for maintaining oral and
dental health and in particular for prevention of the harmful effects of microorganisms. The present study aims to investigate the saliva samples of children with Down’s syndrome (n = 12), mental retardation (n = 11) and autism (n = 7) and compare with the control group (n = 12) cytologically. The study was approved by the local Ethical Committee and financially supported by Grant Project No: SAG-B-130511-0125 from the Commission of Scientific Investigations Projects of Marmara University. The children were between the ages of 6–14 and the control group consisted of healthy children with no disability. Following flushing of mouth with distilled water, unstained whole saliva samples were collected into plastic tubes after 2 h fasting, at 08:00–10:00 a.m. Saliva samples were smeared over a glass microscope slide and fixed with air. Then they were stained with Giemsa stain and microscopically examined (×40) for the presence of epithelium, leucocyte, and bacterium cell counts and the results were evaluated statistically.

SW04.S16–29

Development of multiplex real-time PCR for quantification of cytokines mRNA expression in influenza virus infected human cells
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Cytokines are diverse group of soluble proteins involved in different biological processes, including cell interaction, inflammation, immune response and repair. The analysis of cytokines profile helps to clarify the functional properties of immune cells for research as well as clinical diagnostics. We developed a novel multiplex TaqMan PCR assays for quantification of mRNAs of IL1B, IL2, IL4, IL6, IL10, IL12B, IL18, IFNG and TNF cytokines. Assays include three sets of reactions, in which three cytokines mRNAs and GAPDH mRNA (normalizer) are amplified. Before multiplexing all primer pairs were tested using singleplex PCR at the estimated optimal annealing temperature to check for the correct amplification of the desired fragments. Composition of qPCR mixture was optimized in order to make efficiencies of cytokines mRNAs and endogenous control identical or very close to each other. PCR assays had approximately 100% efficiency. Quantitative multiplex real-time PCR was carried out using CFX96 System (BioRad). The 30 µl mixture contained 1 × Taq buffer (Medigen, Russia), 4 mM MgCl2 (Medigen, Russia), 5 µM Taq polymerase (Medigen, Russia), 0.333 mM dNTP (Promega), well-balanced amounts of forward and reverse primers and TaqMan probes (from 0.15 µM to 0.85 µM). The developed multiplex system was used for measurement of the relative levels of cytokines mRNAs expression in A549 cells, infected with wild type influenza A/California/07/09 (H1N1) vaccine candidate virus at a multiplicity of infection equal to 1. Total RNA was extracted from the control and infected cells after 0.5, 4, 8, 12 and 24 h since infection. At all the time points post infection mRNA levels of IL6 were comparable. It was found approximately 10-fold increase in gene expression of IL1B in cells collected after 24 h of infection. We also observed mild down-regulation of IL18 expression and up-regulation of IL4 expression at 12 and 24 h of infection. IL2 and IFNg were not detected in either infected or mock epithelial cells.

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SW04.S16–30

Amino acid profiling in human follicular fluid and plasma of IVF patients
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Human ovarian follicular fluid (FF) is the in vivo microenvironment for oocyte during folliculogenesis. It is derived from the sanguineous plasma and secretions, synthesised by the granulosa and thecal cells in the follicle wall. FF contains a large variety of growth factors, cytokines, amino acids, and other metabolites. It is reasonable to think that qualitative and quantitative alterations in the composition of the FF affect the quality of the oocyte and hence affect the potential fecundity. FF, being a byproduct of oocyte retrieval and one of the most abundant ‘waste products’ in in vitro fertilization (IVF), represents an optimal source for non-invasive biochemical characterization of oocyte quality. Investigation of the FF composition has increased over the recent years, but its metabolic composition is unfortunately still relatively unknown.

Aim of the current project was to determine the composition of amino acids (AA) that play important roles as osmolytes, intracellular buffers and energy substrates, improving in vitro embryonic development and implantation of embryos in human FF and to compare it with human plasma AA composition.

AA profiling of FF and plasma was performed using a RP-HPLC-UV method. Qualitative and quantitative AA analysis was optimized using commercially available standard compounds. Prior to HPLC analyses, all biological fluid samples were at first treated with AcN in order to precipitate the proteins, followed by derivatization with dapsyl-chloride. For obtained data, wide-ranging statistical analyses were performed. A total of 96 FF and plasma samples from women with different reasons of infertility undergoing consecutive ICSI and embryo transfer at infertility clinic in Tallinn, Estonia, were analyzed. Correlations were tested and comparative analyses of FF and plasma AA concentrations, IVF results and patients' anamnesis were performed. Based on preliminary knowledge and analytical results, potential AA biomarker candidates for assessment of development potential of oocytes were selected.

To the best of our knowledge, this is the most comprehensive amino acid profiling in IVF patients' biological fluids, human follicular fluid and plasma, performed so far.

SW04.S16–31

Non-enzymatic systemic antioxidants as potential biomarkers for chronic obstructive pulmonary disease
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Background: Oxidative stress occurs when the balance between oxidants and antioxidants shifts in favour of oxidants. Our aim
was to compare levels of non-enzymatic antioxidants in sera of patients with stable chronic obstructive pulmonary disease (COPD) and healthy subjects, and to evaluate their role as potential biomarkers for COPD.

**Materials and methods:** The study included 45 healthy subjects and 106 stable COPD patients. COPD patients were additionally subdivided according to disease severity (GOLD stages II-IV) and smoking habits. FEV$_1$ predicted and FEV$_1$/FVC were determined by spirometry. Ceruloplasmin, albumin and transferrin concentrations were determined in serum samples using an automated analyzer.

**Results:** We observed increased ceruloplasmin concentrations in patients compared to controls (0.599 ± 0.266 vs. 0.450 ± 0.139, p = 0.001) while albumin and transferrin concentrations were lower in COPD compared to healthy subjects (39.3 ± 5.8 vs. 49.2 ± 6.9, p < 0.001, and 2.29 ± 0.56 vs. 2.92 ± 0.77, p < 0.001, respectively). Smoking habits did not influence levels of antioxidants tested and neither did disease severity. However, significant differences were found in ceruloplasmin, albumin and transferrin concentrations between controls and GOLD II patients. ROC analysis for ceruloplasmin showed modest performance with area under curve (AUC) of 0.68 [95% confidence interval (CI) 0.60–0.75, p = 0.001], diagnostic sensitivity and specificity of 52.8% and 73.3%, respectively, for the cut-off value of >3.019 g/l. Albumin and transferrin yielded good discriminatory power with AUC of 0.87 (95% CI 0.80–0.92, p < 0.001) and 0.78 (95% CI 0.70–0.84, p < 0.001), respectively. A cut-off value for albumin of ≥43.1 g/l yielded 74.5% sensitivity and 86.1% specificity while the cut-off value for transferrin of ≥24.6 g/l yielded 72.6% sensitivity and 77.8% specificity.

**Conclusions:** The observed changes in ceruloplasmin, albumin and transferrin concentrations suggest disturbed systemic antioxidant capacity in patients with stable COPD. Although those non-enzymatic antioxidants are not disease-specific, their alterations were significant as early as in GOLD II stage, indicating their potential use as biomarkers for the early diagnosis of COPD.

**SW04.S16-32**

**Determination of asymmetric dimethylarginine in body fluids by ABSCIEX API 3200 liquid-chromatography mass spectrometry**

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**Objective:** Asymmetric dimethylarginine (ADMA) acts as endogenous competitive inhibitor of endothelial nitric oxide synthase (eNOS) and blocking nitric oxide generation, it initiates and promotes processes involved in some cardiovascular disorders such as atherogenesis and plaque progression, preeclampsia development, renal failure, hypertension and hypercholesterolemia. ADMA plays also the biological relations of ADMA, arginine and citrulline with several pathological conditions, it is of importance to develop a method to separate and quantify these metabolites in biological fluids. Our aim was to determine these metabolites in LC-MS/MS system.

**Material and Methods:** Mass spectrometric analyses were performed using a Shimadzu LC-20 AD coupled with an ABSCIEX API 3200 triple quadrupole mass spectrometer equipped with an electrospray ion source (ESI) operating in positive mode. Analytical column used for separation was Phenomenex Luna C18 (4.6 m, 3 × 100 mm) column. The chromatographic run was performed at 800 μl/min with a gradient elution of a mobile phase and total analysis run time was 5 min. Mass spectrometric parameters were as: Curtain Gas:10, Temperature: 550°C, Gas 1: 60, Gas 2: 60, Interface Heater: ON, Collision-activated dissociation: 5, IS: 5500, Declustering Potential: 40, Entrance Potential: 7.5, Collision Energy: 24, Duration Time: 5 min.

**Results:** Standard curves for ADMA, Arginine and Citrulline was linear within the range of 0.1–4 μM, 10–250 μM and 10–250 μM in water, respectively. The coefficients of correlation (r$^2$) were 0.9983, 0.9934 and 0.9964. The estimated recovery for all analytes ranged between 99 and 116%. For ADMA limit of blank, limit of detection and limit of quantitation levels were 0.017 μM, 0.05 μM, 0.1 μM respectively.

**Conclusion:** The method presented here uses small sample volume for plasma, serum and urine. Arginine and citrulline levels can be determined in the same analysis and useful for routine measurements with high accuracy and precision.

**SW04.S16-33**

**Diagnostic potential of fetal and embryonic hemoglobins as a markers of hypoxia, fetal development and hemoblastosis**


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**Aims:** Detect and level changes of embryonic and fetal hemoglobins (HbE and HbF) in the blood of adults with hypoxia various origins, newborn tissue hypoxia and myeloproliferative bone marrow diseases. If the study of HbF and HbE in hypoxia is absolutely logical, their study of diseases of myeloid population of pluripotent cells based on modern concept that erythremia relates to myeloproliferative diseases of the marrow.

**Methods:** HbE and HbF we studied with the use self-designed immunochromatography test in 285 serum samples of patients with circulatory hypoxia (stable angina, postinfarction cardiocoronary, 135) and respiratory hypoxia (obstructive bronchitis, asthma, 150), in 196 samples of newborn umbilical blood, 96 those of blood donors, 125 samples of hemoblastosis patients (nonspecific groups from 18 to 38).

**Results:** HbF concentration in the blood of patients with circulatory and respiratory hypoxia was higher (p < 0.001) compared with the donors. But the level of HbF in the blood of patients with ischemic heart disease was 3.7 times higher, and in the group of patients with obstructive bronchitis and asthma in 2.7. Protein level also depends on age, severity and duration of disease. HbF levels in the newborn depends on the severity of hypoxia. It is in 1.3–1.7 times higher than in healthy newborns. The girls fetal hemoglobin found twice as often as boys (39.13 and 20.0% respectively). HbE first registered in the blood of newborns only with severe fetal hypoxia (30.23%). HbF levels were significantly higher in myeloproliferative diseases (erythremia – 1.8, myeloid leukemia various forms 1.4–1.6) than in healthy patients. HbE first identified in patients with erythremia (66.67%), and myeloid leukemia (49.41% – subacute form, 24.44% – acute form, 20.53% – chronic form). HbF, as HbE, was not detected in subacute and chronic lymphocytic leukemia.

**Conclusions:** Embryonic and fetal hemoglobins not only indicators of hypoxia of different genesis in adults, but also the possible additional markers assessment of the risk of adverse neonatal and early neonatal period, and the monitoring of treatment hemoblastosis. Resynthesis antenatal hemoglobins in adults is an example of differential gene activity influenced by pathogenic factors.
SW04.S16–34
Identification of genes of bacterial enzymes beta-lactamases on silicon microchips using gold nanoparticles as a label
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A method for identifying genes and mutations in them by hybridization analysis on silicon microchips using gold nanoparticles as a label was developed. Oligonucleotide probes were immobilized covalently on silicon microchips. DNA duplexes were formed on the surface of the microchip in a result of the interaction of complementary oligonucleotide probes and DNA labeled by biotin. The duplexes then were developed with a conjugate of streptavidin with gold nanoparticles. The size of the gold nanoparticles was 25 ± 3 nm. The nanoparticles on the microchip surface were identified by scanning electron microscopy (SEM) with an electron microscope SUPRA 40 (Carl Zeiss).

The method was applied for identification the genes of bacterial enzymes beta-lactamases. These enzymes cause hydrolysis of beta-lactam antibiotics and confer bacterial resistance to beta-lactam antibiotics.

A feature of the method is to record the number of DNA duplexes on a surface by the number of gold nanoparticles revealed by SEM. To calculate the number of DNA duplexes the fragments of 1.7–30 µm² area were used. Spots not containing immobilized probes or containing immobilized probes with non-complementary structure were used as control spots. The registration by number of single DNA interactions on the microchip was compared with detection of biotin-labeled DNA by means of enzymatic colorimetric detection. The advantages of the method based on nanoparticles counting consist in higher sensitivity by 2.5 orders of magnitude and better signal/background ratio.

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SW04.S16–35
A set of DNA microarrays for rapid determination of bacterial resistance towards beta-lactam antibiotics
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The production of β-lactamases is the predominant cause of resistance to β-lactam antibiotics in Gram-negative bacteria. To date, more than 700 β-lactamases have been described. Among them extended-spectrum β-lactamases (ESBLs) are the most widespread and clinically significant enzymes which are active against practically all penicillins and cephalosporins. During last years the clinical value of carbapenemases of molecular classes A, B and D increased noticeably. So far as many clinical isolates produce more than one β-lactamase and due to the high diversity of these enzymes their determination at the molecular level ensures adequate information for the identification of antimicrobial resistance.

In the present research we have developed several types of oligonucleotide microarrays with horseradish peroxidase (HRP) – based detection for the identification of genes encoding ESBLs and carbapenemases of A, B and D classes. Specific oligonucleotide probes were designed to determine β-lactamase type and important mutations responsible for the broadening of substrate specificity or resistance to inhibitors. The method of DNA microarray consisted of several steps involving DNA extraction, amplification and labeling of target DNA with biotin by two multiplex PCRs and the subsequent hybridization of a PCR product with specific oligonucleotide probes immobilized on glass slides, 96-well polystyrene plates or membrane supports. After hybridization biotin in DNA duplexes was developed with the streptavidin–HRP conjugate followed by colorimetric detection of the enzyme. Careful optimization of oligonucleotide probes and hybridization conditions ensured specific identification of all control isolates producing ESBLs and carbapenemases in a single array. The method developed has been applied successfully for the detection of ESBL and carbapenemase genes in a series of clinical isolates of Enterobacteriaceae, Pseudomonas spp. and Acinetobacter spp.

DNA microarray technique offers the identification of a pathogen antibiotic resistance at the molecular level and is proposed as a useful tool for epidemiological investigation of ESBL- and carbapenemase-producing microorganisms.

SW04.S16–36
Highly sensitive and specific real-time PCR assay for the detection of proteolytic bacterial toxins
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Anthrax is dangerous infectious disease caused by the encapsulated, spore-forming bacterium Bacillus anthracis. Systemic forms of the disease, such as inhalational anthrax, are characterized by nonspecific early symptoms, rapid progression, and, if untreated, by lethality approaching 100% and poor prognosis even with antibiotic treatment. Lethality of anthrax is due to accumulation of lethal toxin (LeTx), comprised by protective antigen (PA), and lethal factor (LF), a zinc metalloprotease with pleiotropic and incompletely understood mechanism of action. No therapy is available to block LeTx intoxication inside the cell. Early anthrax detection is essential for timely initiation of treatment. In the present study, we focused on development of ultrasensitive detection of LF using a modification of the immuno-PCR assay.

The key element of the new detection system is the recombinant protein conjugated with a ss oligonucleotide at a specific site. The protein consists of the N-terminal GST moiety and the in vivo biotinylated peptide moiety separated by the efficient LF cleavage site, RRKKYPYPME. The NH2-modified oligo is attached to the C-terminus of the protein via single engineered Cys residue by using SDP2 heterobifunctional crosslinking agent. Cleavage of the protein-oligo construct immobilized on the glutathione Sepharose, released C-terminal biotinylated peptide-DNA conjugate that is further captured by neutravidin-coated magnetic beads. Captured conjugate is amplified by real-time PCR. Double capture strategy efficiently suppressed nonspecific signal due to DNA absorption. LF was isolated from samples by using mAb, specific to the PA-binding domain, and displaying no interference with LF cleavage activity. Isolated LF was mixed with the conjugate to initiate cleavage.

The detection limit of the developed assay <2 pg of LF in the model experiments with the toxin component titrated in water solutions, and <10 pg of LF isolated from LF-spiked plasma samples. No nonspecific amplification was observed in control
samples subjected to the double capture technique. The developed assay can be used in clinic for prevention of lethality in patients with systemic anthrax infection.

**SW04.S16–37**  
**Determination of oxidative stress, antioxidant status and inflammation in patients with recurrent oral aphthous ulcers**  
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Recurrent aphthous ulcer (RAU) is an inflammatory condition of the oral mucosa characterized by painful, well circumscribed, single or multiple round or ovoid ulcerations. The exact etiologic factor(s) of these ulcerations are not yet understood. These factors have a direct or indirect potential for disturbing the equilibrium between oxidant and antioxidant systems of the organism. To evaluate oxidative stress, antioxidant status and cytokine determined in saliva and plasma of patients with recurrent aphthous ulcer. The study involved 25 patients with RAU and 25 healthy controls. Total antioxidant status (TAS), total oxidant status (TOS), nitric oxide (NO) levels were assessed in saliva and plasma. TOS level were increased in the plasma of RAU patients (p = 0.01, respectively) while, TOS, IL-12, NO, interleukin-12 (IL-12), myeloperoxidase (MPO), NO, interleukin-12, (IL-12), levels were decreased (p = 0.049, p = 0.01) respectively. In saliva, TOS, IL-12, 12, IL-12, IL-12, NO, interleukin-12, (IL-12), levels were assessed in plasma. TOS level increased in the saliva of RAU patients (p = 0.013, p = 0.011 respectively). TOS levels were decreased (p = 0.049, p = 0.01) while, TOS, IL-12, levels were increased (p = 0.027, p = 0.046, p = 0.001, p = 0.042; p = 0.023, p = 0.037, p = 0.01, respectively) in the plasma of RAU patients. Oxidative damage, antioxidant defense and abnormal cytokine expression are important aspects in RAU.

**SW04.S16–38**  
**Evolution of lipidomics in health and disease: the lipid droplet in the center**  
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At the far end of genomics/transcriptomics and proteomics, lipidomics has evolved to cover quantitatively the spatial and temporal distribution of all lipids in a biological entity. The methods of choice are LC-hyphenated and shot-gun mass spectrometry (MS), which allows profiling of lipid species and structural elucidation of lipid molecular species. The former offers determination of phenotypes and insights into correlations desired in clinical settings, the latter aims to unravel structure-function relationships and metabolic paths in healthy and diseased states enabling determination of biomarkers or targeted drug research. These aspects will be demonstrated by studying lipid droplets (LDs) which are composed of mainly triacylglycerols (TGs) in the hydrophobic core and of the surrounding phospholipid monolayer. The focus is on hepatocyte LDs from mouse models exposed to nutritional stress (high fat diet, starvation) and to genetic stress generated by deficiency of adipocyte triglyceride lipase (ATGL) or its co-activator comparative gene identification protein 58 (CGI-58). These two proteins are needed to initiate cellular lipolysis at the LD surface, the rate limiting step for TG degradation. The liberated fatty acids are subsequently targeted to β-oxidation and membrane biosynthesis, or can have regulatory functions. Mutations in the ATGL gene lead to ‘Neutral lipid storage disease (NLSD) with myopathy’ and mutations in the CGI-58 gene to ‘NLSD with ichthyosis’, both associated with fatty liver. By using an LC-MS/MS platform, together with bioinformatic tools, we were able to demonstrate that the various stresses applied to the mouse models during intervention studies resulted in phenotypic imprints on TG, diacylglycerol (DG) and partly phophatidylcholine (PC) lipides in hepatocyte LDs. Moreover, TG biomarkers were identified for pathophysiological situations with or without insulin resistance and structures of lipid molecular structures revealed prominent metabolic relationships.

**SW04.S16–39**  
**New potential molecular targets to treat ocular diseases**  
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Diabetic retinopathy and glaucoma are two non-curable retinal degenerative diseases, which are leading causes of vision loss and blindness worldwide. Diabetic retinopathy affects retinal microvasculature, but increasing evidence also shows that the neural components of the retina are affected as well. Glaucoma is characterized by the degeneration of retinal ganglion cells and optic nerve. Regarding diabetic retinopathy, the treatments available are scarce, being mostly directed for the later stages of the disease, having low efficacy. In glaucoma, there are more therapeutic options, which are mainly directed to control the intraocular pressure (IOP), since increased IOP is a major risk factor. However, despite good IOP control, the disease progresses and new treatments are needed. Neuroprotection has been viewed as a potential additional strategy for the treatment of glaucoma, but also for diabetic retinopathy. In the last decade, increasing evidence has also shown that both diseases can be considered low grade chronic inflammatory diseases. We have shown that the inducible nitric oxide synthase (iNOS) isoform has a predominant role in the breakdown of blood-retinal barrier and that the atypical isoforms of protein kinase C (PKC) can be viewed as potential targets to treat retinal diseases characterized by increased vascular permeability. Finally, we have found that neuropeptide Y (NPY), a neuromodulator that is involved in many physiological functions, has potent neuroprotective properties against retinal cell death, and is also able to reduce neuroinflammatory processes in the retina mediated by activated microglial cells.

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**SW04.S16–40**  
**Type 1 diabetes affects expression of 14-3-3 proteins in a tissue specific way**  
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Type-1 diabetes (T1D) is an endocrine disorder caused by autoimmune destruction of pancreatic β cells, leading to uncontrolled chronic hyperglycemia. Complications related to T1D result in
the dysregulation of several cellular pathways leading to target organ damages like diabetic cardiomyopathy, hepatic and renal failure and diabetic encephalopathy [1]. A most used animal model of T1D is obtained by the administration of streptozotocin (STZ) to rats, thus giving the opportunity to tightly monitor the onset of the disease. The 14-3-3 family proteins are key signaling molecules, involved in the regulation of multiple cellular pathways, mainly the apoptotic one, consisting of seven distinct isoforms (β, γ, ε, ζ, τ, η, σ), mainly localized in the cytosol.

In this study we investigated the expression of 14-3-3 family proteins at transcript and protein levels in the brain and liver of short-term T1D rats.

T1D was induced by STZ treatment and after 3-weeks purified brain and liver cytosolic proteins were prepared from control and T1D rats [2]. The protein expression of all the seven 14-3-3 isoforms were studied by Western blot analysis using high-specific monoclonal antibody and the relative transcript levels were assessed by Real-time quantitative PCR. At brain level all the 14-3-3 isoforms showed a statistical significant changes in protein expression and mRNA level in T1D sample compared to control ones. In detail, six of the seven 14-3-3 isoforms (β, γ, ε, ζ, τ, η) showed a marked decrease of both protein and mRNA content in the T1D brain, while for the 14-3-3 σ isoform the protein decreased level was opposite to the increased mRNA content in T1D rats. This finding could be attributed to post-translational events or to an enhanced binding of the protein with one of its multiple targets.

On the other hand, in the liver of T1D rats only one 14-3-3 isoform (γ) showed a slight statistical significant change, showing in this case an increment in both protein content and mRNA level. Overall our results indicate that the impact of short-term T1D on 14-3-3 proteins and transcript expression is different at brain and hepatic level. This might be associated with activation or inactivation of apoptotic pathways in a tissue-specific way.

References

SW04.S16-41
Glycation impairs albumin drug binding properties in diabetic patients
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Glycation is a biological mechanism whose enhanced occurrence in diabetes has been involved in the pathology progression. Albumin is the major circulating protein and exerts many beneficial activities in blood. One of the main properties of this plasmatic protein is to bind hydrophobic molecules. So, albumin plays a pivotal role in therapeutic actions for hydrophobic drugs such as warfarin and ketoprofen.

In hyperglycemic condition, albumin undergoes enhanced structural modification by glycation, which impacts the protein properties.

In this study, we investigated the impact of in vitro and in vivo glycation on albumin drug binding capacities. For this, we compared structural and functional properties of albumin purified from diabetic patients with in vitro glycated albumin models.

Glycation-induced modifications on human serum albumin (HSA), including redox state and ketoamine contents, were investigated in parallel with HSA binding to Warfarin and Ketoprofen. High-performance liquid chromatography (HPLC) was used to determine the free drug concentrations and dissociation constants according to the Scatchard method.

Oxidation and glycation levels were found to be enhanced in albumin purified from diabetic patients or glycated with glucose or methylglyoxal, after determination of their ketoamine, free thiol, amino group and carbonyl contents. In parallel, significant impairments in the binding affinity of in vitro and in vivo glycated albumin were observed, as indicated by the higher dissociation constant values and confirmed by higher free drug fractions.

Even if our work needs further investigation, we propose that albumin redox state could contribute to variability in drugs response during diabetes. This work provides new evidence supporting in vivo diabetic albumin could represent a very useful model of glycation for monitoring diabetic physiopathology.

SW04.S16-42
Novel mechanism of L-lysine alpha-oxidase biological action
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Compared to normal cells, cancer cells are more sensitive to the deficiency of growth factors, including amino acids, which play an important role in metabolism. L-lysine is an essential amino acid and a potential molecular target of anticancer agents. The search for L-lysine depleting agents led to the discovery in Trichoderma fungi of L-lysine alpha-oxidase (LO) (EC1.4.3.14), which catalyses l-lysine oxidative deamination. Besides inhibition of protein synthesis, LO causes numerous biological effects: remarkable decrease of DNA synthesis and cell transformation from S to G2/M phase, change of cellular membrane permeability (demonstrated by penetration of 3H-inosine into hepatoma AG-22 cells), decrease of the number of ß-adrenergic receptors and cell adhesion proteins on the cell surface (shown in leukosis P388 cell line), anti-inflammatory and anti-metastatic effect.

After intravenous injection of LO at 1 mg/kg L-lysine level in serum was negligibly low even 24 h after injection. LO is also capable of processing L-lysine structural analogs, L-arginine and L-ornithine, which are the precursors of polyamines (PA), however the efficacy of LO towards these substrates is <6% of that towards l-lysine. The goal of the present work was to determine the ability of LO to influence PA metabolism. LO was administered to mice intravenously at 1.0 mg/kg. Three animals per time point were sacrificed at 10, 30 and 60 min after administration. Brain and liver were snap-frozen in liquid nitrogen. The content of PA in the samples was measured using high-performance liquid chromatography with two repeats per sample.

Marked reduction of PA levels compared to controls was observed 10 min after LO administration. In liver the putrescine level decreased by 63%, spermidine – by 35%, spermine – by 22%. Later at the constantly low putrescine level the concentrations of spermidine and spermine further dropped down to 50% of the control. In brain the effect of LO on PA levels was less pronounced than in liver.
The observed PA level decrease after LO administration found in the present study allows new insights into the molecular mechanism of LO activity in vivo, which might be responsible for some of the pharmacological effects of LO, including anti-tumor activity.

**SW04.S16–43**

**Pyrimethamine chaperone enhances beta-hexosaminidase activity in Sandhoff fibroblasts without restoring lysosomal GM2 catabolism**

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Beta-hexosaminidase activities were tested on fibroblasts from two patients showing symptoms characteristic for GM2 gangliosidosis, but with a slower clinical evolution compared to classical infantile forms. These biochemical analyses performed using artificial substrates confirmed not only the diagnosis of Sandhoff disease, but also the existence of a possible residual activity. As pyrimethamine has recently been described as a new pharmacological chaperone for GM2 gangliosidoses, the efficacy of this component was evaluated in both patients. Unfortunately, even if an increase of the residual activity was observed with the artificial substrate, this was not confirmed using GM2 natural substrate. Our results highlight the fact that a particular attention should be paid to the use of this new molecular therapeutic approach in patients and that feeding experiments with tritium-labeled gangliosides on patient fibroblasts are useful to determine the effect of pyrimethamine on the recovery of lysosomal β-hexosaminidase activity. Our report confirms that only some mutant proteins are candidate to a chaperone treatment with pyrimethamine and it was not the case for our two patients. The clinical chemistry methods used in our study give a valuable information to decide on the therapeutic use of pyrimethamine in patients.

**SW04.S16–44**

**Novel causative relationship between low HDL and diet-induced nonalcoholic fatty liver disease**

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During the biogenesis of HDL, lipid free or minimally lipidated apoA-I interacts functionally with the lipid transporter ABCA1 to form immature discoidal HDL which are then converted into mature spherical particles by the action of lecithin:cholesterol acyl transferase (LCAT). Here we investigated the mechanistic relationship between low and dysfunctional HDL and diet-induced NAFLD development using mouse models. In our current study, we employed male apoA-I-deficient (apoA-I−/−) mice that lack classical apoA-I containing HDL and male deficient (LCAT−/−) mice that have immature discoidal HDL. Mice were fed the standard western-type diet for 24 weeks and then histological and biochemical analyses were performed. ApoA-I−/− mice showed increased diet-induced hepatic triglyceride deposition and disturbed hepatic histology while they exhibited reduced glucose tolerance and insulin sensitivity. Quantification of FASN-1, DGAT-1, and PPARγ mRNA expression suggested that the increased hepatic triglyceride content of the apoA-I−/− mice was not due to de novo synthesis of triglycerides. Similarly, metabolic profiling did not reveal differences in the energy expenditure between the two mouse groups. However, apoA-I−/− mice exhibited enhanced intestinal absorption of dietary triglycerides, accelerated clearance of postprandial triglycerides, and a reduced rate of hepatic very low density lipoprotein triglyceride secretion. In agreement with these findings, adenosine-mediated gene transfer of apoA-I Milano in apoA-I−/− mice fed western-type diet for 12 weeks resulted in a significant reduction in hepatic triglyceride content and an improvement of hepatic histology and architecture. Similar to apoA-I−/− mice, LCAT−/− mice were characterized by increased diet-induced hepatic triglyceride deposition and impaired hepatic histology and architecture. Adenosine-mediated gene transfer of LCAT in LCAT−/− mice that were fed western-type diet for 12 weeks resulted in a significant reduction in hepatic triglyceride content and a great improvement of hepatic histology and architecture. Taken together, our data establish that the HDL metabolic pathway is a central contributor to the deposition of dietary triglycerides to the liver and the development of NAFLD. Our data further support that the coexistence of reduced HDL levels and NAFLD in an individual with metabolic syndrome may not be a mere coincidence, rather it underlies a strong causative relationship between these two conditions.

**SW04.S16–45**

**APOB and ABCA1 gene polymorphisms in Bosnian patients with type 2 diabetes**

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Dyslipidemia represents a risk factor for type 2 diabetes (T2D) and cardiovascular disease. Recent genome-wide association studies have identified multiple single nucleotide polymorphisms (SNPs) associated with plasma lipid concentrations. In this study, we examined the associations of variants in two candidate loci: rs693 in apolipoprotein B gene (APOB) and rs3890182 in ATP-binding cassette transporter A1 gene (ABCA1), with T2D and metabolic traits in a population from Bosnia and Herzegovina. APOB and ABCA1 play a major role in the metabolism of low-density lipoprotein (LDL) and high-density lipoprotein (HDL), respectively.

Subjects were genotyped for rs693 (50 T2D patients and 51 nondiabetic controls) and rs3890182 (61 patients and 75 nondiabetic controls) SNPs, using the MassArray Sequenom iPLEX platform. There was a significant difference in genotype frequencies for APOB rs693 variant between T2D patients and nondiabetic controls (p = 0.011). The mutated allele frequency was 33% and 50% in diabetic and nondiabetic subjects, respectively. The rs693 SNP was associated with 3-fold decreased risk for T2D (dominant model, adjusted OR = 0.33, 95% CI = 0.14–0.79, p = 0.013). It also showed an association with lower fasting plasma insulin levels in T2D patients (p = 0.003). In controls, mutant/variants and carriers had significantly lower fasting glucose levels compared to the G allele carriers (p = 0.046). Significant associations of genotypes with lipid concentrations were not found. The genotype distribution for ABCA1
rs3890182 SNP was not significantly different between T2D patients and controls. The variant A allele of rs3890182 SNP showed a significant association with lower HDL-cholesterol (HDL-C) levels in nondiabetic subjects (p = 0.013). In conclusion, our preliminary study replicated ABCA1 and HDL-C association in a small Bosnian cohort. Our data suggested a protective effect of APOB rs693 SNP in T2D, which should be further explored in larger cohorts.

**SW04.S16–46**

**Testosterone deficiency reduces diet-induced weight gain, energy expenditure, and glucose intolerance in mice lacking the low density lipoprotein receptor**

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Despite the role of the lipoprotein system in the management of dietary lipids in circulation, the functional crosstalk between testosterone and lipoprotein metabolism in the development of diet-induced obesity remains vaguely defined. Based on our previous results showing that the chylomicron metabolic pathway possesses a central role in the development of obesity and related metabolic abnormalities, here we sought to determine how low of testosterone affects obesity, energy expenditure, and glucose metabolism in castrated low density lipoprotein receptor deficient (LDLr−/−) mice, a model of familial homozygote hypercholesterolemia. Though sham operated animals fed western-type diet for 12 weeks became obese and showed decreased energy expenditure, disturbed plasma glucose metabolism and increased plasma cholesterol and triglyceride levels, castrated LDLr−/− mice appeared resistant to diet-induced obesity, had elevated energy expenditure, improved glucose metabolism and reduced plasma triglyceride levels, though they presented a significantly deteriorated plasma cholesterol profile. Based on previous findings showing that apoE expression levels modulate obesity and glucose metabolism in the present study we tested the hypothesis that reduced apoE expression are responsible for the observed phenotype in hypogonadal LDLr−/− mice. Western blot analysis of plasma from control and orchoectomized mice showed that TD does not affect apoE protein levels in these mice suggesting that a reduction in plasma apoE levels cannot account for the observed phenotypic differences between sham and castrated mice. Our data have unexpectedly revealed a functional cross-talk between testosterone and apoE that modulates processes associated with metabolic syndrome though the mechanism mediating the observed differences needs further investigation.

**SW04.S16–47**

**LVVYPW stimulates beta-endorphin secretion to lower plasma glucose in streptozotocin-induced diabetic rats**

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Recently, we have demonstrated the blood glucose-lowering effect of LVVYPW, a member of hemorphins family, in streptozotocin (STZ)-induced diabetic rats. The involvement of µ-opioid receptors (MOR) in the molecular mechanisms of anti-diabetic effect of LVVYPW was shown by using MOR antagonists naloxone and naloxonazin. In present study we have revealed that, in parallel to plasma glucose-lowering effect, LVVYPW enhances the level of β-endorphin, reduced in the plasma of diabetic rats. Determination of plasma β-endorphin level was performed by using Beta-Endorphin (Rat) ELISA kit (Phoenix Pharmaceuticals, Inc., USA) according to manufacturer’s recommendations. The intraperitoneal injection of LVVYPW (1 mg/kg) into fasting STZ-induced diabetic rats (male, Wistar line, weighing 180–220 g) completely recovered plasma β-endorphin level. Thus, data obtained represent an additional evidence concerning the involvement of MOR in the molecular mechanisms of anti-diabetic effect of LVVYPW. It is necessary to emphasize that activation of MOR, induced by the increased β-endorphin secretion, results in an increased expression of insulin-responsive glucose transporter 4 (GLUT4) and this is one of the mechanisms of action of anti-diabetic drugs, including metformin.

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**SW04.S16–48**

**Modulation of Cox-2 and iNOS expression in macrophages by a lipophylic extract of the sea-star Marthasterias glacialis: cooperative effect of fatty acids and ergosta-7,22-dien-3-ol**

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In this study, the anti-inflammatory activity of a purified fraction of the sea-star Marthasterias glacialis lipidome on macrophages was investigated. GC-MS analysis revealed the presence of fatty acids (saturated and unsaturated) and sterols, some of them described in this species for the first time.

LPS treatment elicited loss of cell viability and an increase in the levels of NO, IL-6 and intracellular ROS, all of which were ameliorated by pre-incubation with the extract in a concentration of 156 µg/ml. In a similar way, the LPS-induced increase in the protein levels of COX-2 and iNOS was prevented by the extract.

The pro-inflammatory effects of LPS were also extended to the endoplasmatic reticulum, which resulted in the increase of the expression of CHOP, a reticular stress protein marker, an effect partially prevented by the extract.

Taking into account the findings regarding the chemical composition of the purified extract, the compounds responsible for the activity displayed were investigated.

Overall, the combination of palmitic acid, cis 11-eicosanoic acid, cis-11,14 eicosadienioic and the sterol ergosta-7,22-dien-3-ol were able to mimic the anti-inflammatory effect of the extract, an effect in which the sterol plays a major role.

This work is the first report on the anti-inflammatory activity of ergosta-7,22-dien-3-ol in this inflammation model and establishes the interest of M. glacialis as a source of bioactive molecules.

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Second generation antipsychotic (SGA) drugs modify the differentiation program of human adipocytes inducing ‘browning’ markers

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Although brown adipose tissue (BAT) can be found only in small amounts in the human body after infancy, recent studies revealed that it has a major importance in regulating the energy balance of the entire body contributing to energy expenditure during cold exposure and diet or physical exercise induced thermogenesis. Antipsychotic drug treatment at clinically administered concentrations reprogrammed the gene expression pattern of differentiating human adipocytes, surprisingly leading to overexpression of the major BAT marker gene, UCP1. Our aim was to clarify if a commonly used second generation antipsychotic drug (SGA) was able to induce a browning program on differentiating human adipocytes. Furthermore, we intend to establish an in vitro model of human brown adipocyte differentiation and to set up a panel of measurements that can discriminate between white and brown adipocytes including determination of expression of white and brown adipocyte markers by RT-QPCR, immunoblotting and changes in morphology or mitochondrial properties by Laser Scanning Cytometry. Human preadipocyte cell line or primary preadipocytes obtained from herniotomy were differentiated into white [1] or brown [2] adipocytes with or without SGA treatment. SGA administration resulted in significant overexpression of several brown adipocyte marker genes (UCP1, ELOVL3, CIDEA, CYC1, PGC1A) while SGA treated cells had more and smaller lipid droplets than the control ones. Although, SGA treatment can induce a browning program in differentiating human white adipocytes in vitro, the background of weight gain, its common side effect has not been elucidated yet.

References

SW04.S16-50
Prostaglandin E(1) reduces renal ischemia/reperfusion-induced gastric damage through its anti-inflammatory and anti-oxidative effects

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Gastrointestinal complications including peptic ulcer are frequent in renal transplant recipients. These disorders may be related to infections and/or exacerbation of pre-existing gastric pathology. In this regard, renal ischemia/reperfusion-induced gastric damage seems important and there is not any data about the mechanism of this pathology. The aim of this study is to investigate the possible role of prostaglandin E(1) in renal ischemia/reperfusion-induced gastric damage by microscopically and biochemically. In this study, Sprague Dawley male rats were randomly divided into four groups. First group was generated control rats given生理logic salt solution; second control group was generated rats given only prostaglandin E(1) (20 µg/kg); third group was experimental rats carried out renal ischemia/reperfusion model; and fourth group was experimental rats given prostaglandin E(1) (20 µg/kg) and applied ischemia/reperfusion model. With prostaglandin E(1) pretreatment to renal ischemia/reperfusion model applied rats the following results were noted: (i) Gastric damage was reduced; (ii). The number of cleaved caspase-3’ epithelial and inflammatory cell was decreased; (iii). NF-xB-’ inflammatory cell number was decreased; (iv). Proliferative cell nuclear antigen ’ epithelial cell number was increased; (v). ROS production and oxidative damage were decreased. These results suggest that renal ischemia/reperfusion-induced ROS production increased inflammation through NF-xB activation in inflammatory cells. Then inflammation triggers apoptosis and suppressed proliferation in epithelial and inflammatory cells. According to our data we can suggest that prostaglandin E(1) reduces renal ischemia/reperfusion-induced gastric damage through its anti-inflammatory and anti-oxidative effects.

SW04.S16-51
Impairment of signaling pathways in skeletal muscle of chronic alcohol consumers

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Chronic alcoholic muscle disease is one of most frequent and profound manifestations of chronic alcohol intoxication. This disease is characterized by the pronounced atrophy of the locomotor muscles, which involves predominantly those fibers expressing myosin isoforms of the II ‘fast’ type. At the same time patients at the early stages of the disease development may not always demonstrate signs of fiber atrophy. In earlier experiments with alcohol-fed rats it was shown the impairment of the anabolic intracellular signaling pathways and decrease in protein synthesis rate [Hong-Brown et al, 2012 and others]. However the alterations of the signaling pathways in muscles of patients with alcoholic myopathy are not studied yet. We were the first to analyze the signaling pathways involved in the pathogenesis of alcoholic muscle disease in patients with different fiber atrophy levels. In biopsy samples of m. vastus lateralis (even at the early stages of the disease) we found dramatic drop of the phosphorylation levels of S6 ribosomal kinases and increase in phosphorylation of the eukaryotic elongation factor-2, which facilitate the decrease of the protein synthesis levels. At the early stages we observed also the significant increase of mRNA of E3 ubiquitin ligases. However the total ubiquitinylation level was not altered in patients as compared to the control subjects. This phenomenon could be associated with the increased expression of the heat-shock proteins, known for their protective action. Thus in patients with alcoholic myopathy we revealed the impairment of the anabolic as well as catabolic signaling pathways in skeletal muscle.

The study was performed in collaboration with the Neurological Clinic of the Sechenov First Moscow State Medical University and funded by the Program of the RAS Presidium ‘Basic Sciences for Medicine’.
In this study, male Spraque Dawley rats were used. Rats were randomized into four groups. Group I: control (intact) animals. Group II: control animals administered VOL. Group III: STZ-induced diabetic animals. Group IV: STZ-induced diabetic animals administered VOL. VOL was given to some of the experimental animals by gavage at a dose of 0.2 mM/kg every day for 12 days. Experimental diabetes was induced by single intraperitoneal injection of STZ (65 mg/kg). Blood samples were collected from animals on 0, 1, 6 and 12 days after STZ injection. On day 12, the liver tissues were taken from the animals. The tissue sections were stained with Hematoxylin-Eosin and Masson’s trichrome. In the diabetic group, some degenerative changes such as vacuolization in cytoplasm with dense granular appearance and nuclei including dense chromatin in hepatocytes, endothelial rupture in central veins, hyperemia, dilation in the sinusoids, mononuclear cell infiltration, necrotic areas were observed by light microscopic examination, but the degenerative changes were partly decreased in some animals of the diabetic group given VOL according to diabetic group, although there were individual differences. In the diabetic group, the blood glucose levels and plasma lactate dehydrogenase activity were increased and blood glutathione levels and plasma catalase and glucose-6-phosphate dehydrogenase activities were decreased. The administration of VOL to the diabetic rats reversed these effects. Based on these morphological and biochemical observations, it was concluded that VOL has a curative effect against the hepatotoxicity produced by STZ-induced diabetes.
Elevated level of tumor necrosis factor-α in rats with impaired glucose tolerance

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Type 2 diabetes is very common metabolic disease all over the world, with prevalence constantly increasing over the last few decades. Impaired glucose tolerance is a pre-diabetic state of dysglycemia that may precede type 2 diabetes mellitus by many years. Tumor necrosis factor-α (TNF-α) over-secretion has already been implied in the pathogenesis of insulin resistance and diabetic complications. TNF-α is already found to be increased in both animals and humans with long term diabetes. We aimed to clarify whether TNF-α is increased in rats with impaired glucose tolerance. Ten-week-old male Wistar rats were divided into two groups, A (control) and B (pre-diabetic). Non-insulin dependent diabetes mellitus was induced by intraperitoneal streptozotocin injection following nicotinamide injection. Both groups received standard food for 6 weeks. Fasting serum glucose levels were significantly higher in group B when compared to the group A (group A, 4.81 ± 0.18 mM; group B, 6.51 ± 0.32 mM; p < 0.001). There was no significant difference in insulin among the groups (group A, 1.24 ± 0.11 ng/ml; group B, 1.10 ± 0.09 ng/ml; NS). Serum TNF-α levels were found to be increased in the group B when compared to the group A after only 6 weeks of diabetes onset (group A, 11.27 ± 0.48 pg/ml; group B, 13.21 ± 0.41 pg/ml; p < 0.001). Early application of specific agents that suppress production and/or activity of TNF-α may prevent the development of diabetes type 2.

Complex investigation of familial hypercholesterolemia in North-West Russia

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For the first time we have collected DNA bank from 109 Karelian patients with familial hypercholesterolemia (FH), living in Petrozavodsk and other localities in the Republic of Karelia. By means of automated fluorescent SSCP-analysis, RFLP-analysis and direct PCR product DNA sequencing this collection was searched for presence of mutations in three loci of dominant hypercholesterolemia. We have studied the complete coding sequence of the low density lipoprotein (LDL) receptor gene and performed screening for major mutations in the APOB and PCSK9 genes. We have identified 13 mutations in the LDL receptor gene, namely: p.G20R, c.192del10/ins8, c.195-196insT, p.S206R, c.925-931del7, p.S447C, p.I398I, p.L426P, L511S, c.1686del1/insT, p.L461G, p.N640N, c.2191delG. All mutations were found in unique pedigrees and were not recurrent, what argues against founder effect in Petrozavodsk FH. Out of 13 mutations found 6 were characterized for the first time in the world and 7 were previously reported from other populations. Remarkably, that out of 13 mutations found in Petrozavodsk FH only one mutation, namely c.925-931del7, was common with St.-Petersburg population, where previously also 100 probands with FH were studied. No prevalent mutations both in APOB and PCSK9 genes were detected in Petrozavodsk and in St.-Petersburg FH patient’s DNA collections. It was demonstrated that ‘Finnish’ LDL receptor gene mutations are not typical both for Karelian and St.-Petersburg FH patients. Mutation FH-Hel-sinki was found neither in Petrozovodsk nor in St.-Petersburg population, whereas mutation FH-North Karelia (c.925-931del7) was detected in single family out of 100 studied in both collections. We have studied frequency of the polymorphic markers of the LDL receptor gene, namely p.A391T, p.R471R, p.P539P, p.N591N, p.V653V and p.R744R, in Karelian FH sample. Research was supported in part by RFBR grant 10-04-00563a and 13-04-00902a.

Is there any association between the variants of receptor for advanced glycation end products (RAGEs) and obesity?


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Production and secretion of adipokines from adipose tissue contribute directly to the development of obesity-related complications. However, the underlying mechanism by which obesity leads to dysregulation of adipokine secretion remains to be elucidated. In recent years, studies on advanced glycation end products (AGE) and their interaction with their receptors (RAGE) has increasingly became prevalent and it was reported that AGEs might have potential roles in the development of this mechanism. All characteristic features of obesity such as hyperlipidemia, hyperglycemia and oxidative stress, play important roles in the formation of AGEs. The production of reactive oxygen species, glyoxidation, activation of proinflammatory and prothrombotic pathways were some toxic effects of AGEs. All these effects were driven by binding specific cell surface receptors, RAGEs. The gene for RAGE is located on chromosome 6p21.3 in the major histocompatibility. A functional polymorphism in the promoter of the RAGE gene has been shown to exert significant effects on transcriptional activity. In the present study, examination of the association between obesity and RAGE -374T/A polymorphism was aimed. 50 obese children (29 girl, 21 boy) and 71 normal weight children (38 girl, 33 boy) were included in this study. The genomic DNA was isolated with salting-out procedure and RAGE -374T/A polymorphism was analyzed by PCR based techniques. The allelic and genotypic frequencies were not significantly different between the study groups. The clinical parameters were significantly higher in obese children, 26% of obese children had dyslipidemia, 42% had hypertension, 76% had insulin resistance. The clinical parameters were not differ among RAGE -374T/A alleles or genotypes. However T allele obese carriers (TT+AT) had heavy weight ratio than AA carriers (p = 0.073). Also A allele carriers (AA+AT) had higher blood glucose levels than TT carriers (p = 0.033). Although this report was the first one to determine the relation between RAGE -374T/A and obesity, no association was found. However obesity and having mutant allele with elevated levels of glucose seems to be more risky for the complications of obesity such as diabetes and coronary heart disease.
Deficiency of DPP IV/CD26 impacts vasoactive intestinal peptide levels among the gut-brain axis in acute inflammation

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Inflammatory bowel disease (IBD, including Crohn’s disease and ulcerative colitis), is a group of chronic inflammatory conditions of the gastrointestinal tract with unclear etiology. Increasing scientific evidence confirms a bidirectional connection between central and enteric nervous systems, where peptidases exert a key role in maintaining the homeostasis in the gut via their bioactive substrates. Dipeptidyl-peptidase IV (DPP IV/CD26) is an intrinsic membrane glycoprotein found also in soluble form in biological fluids, associated with different important processes, including immune regulation. Vasoactive intestinal peptide (VIP) is an important substrate of DPP IV/CD26, which involvement in chronic inflammatory processes, including IBD, has been proven. Our hypothesis was that DPP IV/CD26 plays an important role in IBD pathogenesis by influencing circulating and tissue levels of VIP in a chemically-induced model of IBD in mice. In order to evaluate the effect of DPP IV/CD26 on VIP levels among the gut-brain axis, a trinitrobenzenesulfonic acid (TNBS)-induced (Crohn-like) model of colitis has been induced in CD26 deficient and wild type mice. Results of our study showed that CD26 deficient mice constitutionally have statistically significantly (p < 0.05) higher values in the acute phase of colitis, but the increment is more pronounced in CD26 deficient mice. VIP concentrations in serum of both mice strains reach statistically significantly (p < 0.05) higher values in CD26 deficient mice. Changes at the local site of inflammation influenced VIP levels in the brain, also showing increased concentrations in both mice strains in acute inflammation, with statistically significantly (p < 0.05) higher values in the CD26 deficient mice. Our results indicate and prove the importance of the gut-brain axis in the pathogenesis of IBD as well as an important role in maintaining the homeostasis in the gut via their bioactive substrates.

Biochemical and immunological parameters in dynamics under esophageal alkali burn model of 1st and 2nd degrees

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Exogenous poisoning with alkalis takes the leading position among causes of acute poisoning. About 75% of the affected persons are children. There are many pathologies and complications following esophageal burns: scar stricture, esophageal deformations, corrosive esophagitis, gastroesophageal reflex and others. Nowadays there are not enough adequate experimental models of esophageal alkali burns for investigation of this pathogenesis. Thereby the burn disease is an important issue that needs solution immediately.

The purpose of the work was to reproduce the model of an esophageal alkali burn of 1st and 2nd degree experimentally in rats and to characterize the biochemical and immunological blood parameters in dynamics. In experiments, we used nonlinear white mature rats. The animals were administered with NaOH (10% and 20% solutions) to induce esophageal burn. Rat blood samples were obtained after 1, 3 and 7 days after alkali administration.

The biochemical parameters were determined with analyzer Humalyzer 3000. The level of the blood antibodies was assessed by ELISA analysis. The level of circulating immune complexes (CIC) in serum was determined by precipitation with 4.5% solution of polyethylene glycol-6000.

After simulation of an alkali burn of 1st degree the level of investigated biochemical parameters (content of total protein, albumin, urea, creatinine; activities of alanine aminotransferase and aspartate aminotransferase) was varied maximally on the 7th day of research. In the case of 2nd degree burn model these parameters were shown to change mainly on the 1st day of research. The most pronounced pathological process was observed in the group of animals with 2nd degree model burn (increase of IgG antibodies level on 1st day). In the group of animals with 1st degree of esophageal burn, the level of CIC was increased on the 7th day. Thus, the most pronounced pathological process was observed after 2nd degree alkali burn simulation.

So in the experiments, we reproduced an alkali esophageal burn model of 1st and 2nd degrees in rats. The changes of basic blood biochemical and immunological parameters in experimental animals with alkali burn of esophagus were established. Obtained results allow us to conclude that the alkali model used is adequate for human esophageal burns research. Our approach can be used for study molecular mechanisms of alkali esophageal burn pathogenesis of 1st and 2nd degrees.

Effects of specific cyclooxygenase-1 A-842G/C50T gene variation on type 2 diabetes mellitus

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Background: The importance of clinic heterogeneity and genetic factors in type 2 diabetes mellitus has been pointed out for a long time. Recently, epidemiologic and molecular studies have shown evidence on genetic factors playing efficient role on the disease progression. Especially inflammation and homeostasis related genetic factors such as cyclooxygenase efficiency is widely studied. Cyclooxygenase (COX) enzymes catalyze the generation of prostanoids from arachidonic acid. A member of this enzyme family COX-1, catalyses prostaglandin generation from arachidonic acid, is constitutively expressed. Several genetic variations have been described in COX-1 locus such as the C50T polymorphism causing the Pro17Leu (P17L) substitution in exon 2. The A-842G and C50T polymorphisms, specifically determined with full linkage analysis, are associated with decreased acetylsalistic acid (aspirin) efficiency which was shown to be caused by increased tromboxane A2 synthesis capacity and thrombosis aggregation compared to the wild type. In this study, we aimed to determine the effect of COX-1 A-842G/C50T polymorphism on type 2 diabetes and also the distribution of the variation in Turkish population.

Material and Methods: With this perspective, in this study, two groups were included which were comprised of 98 type 2 diabetes patients and 96 healthy controls. In order to determine the COX-
Vanadium and experimental diabetes

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Vanadium is a trace element present in low concentrations in human and mammalian tissues. Various compounds of vanadium have been demonstrated to lower plasma glucose levels in experimental models of diabetes as well as in a number of studies in human diabetic subjects. It was postulated that vanadium compounds exert this effect by their insulin-mimetic activity, therefore these compounds can be proposed as potential antidiabetic drugs.

In diabetes oxidative stress results in the increase of free radicals, leading to cell damage which is one of the main causes of diabetic complications. Though nephropathy, neuropathy and retinopathy are the major diabetes derived disorders, other tissues are also affected.

In different studies undertaken in our laboratories, the effect of vanadyl sulfate supplementation on the antioxidant system in the muscle, lung and stomach tissues of diabetic rats was investigated. Diabetes was induced by i.p. injection of streptozotocin (STZ, 65 mg/kg b.w.) to male Swiss albino rats. The rats were randomly divided into four groups: Group I, control; Group II, vanadyl sulfate control; Group III, STZ-diabetic untreated; Group IV, STZ-diabetic treated with vanadyl sulfate. Vanadyl sulfate (100 mg/kg) was given daily by gavage for 60 days. At the last day of the experiment, rats which were fasted overnight were sacrificed; muscle, lung and stomach tissues were taken, homogenized in cold saline to make 10% (w/v) homogenates. In order to evaluate the effect of vanadium on the antioxidant system, antioxidant enzymes, catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, glutathione-S-transferase, as well as carbonic anhydrase activities were determined.

All of the enzyme activities were increased in the diabetic groups and it was shown that vanadyl sulfate administration decreased significantly enzyme activities in all groups. Vanadium did not exert any effect on tissue antioxidant enzyme activities when given alone to normal rats. As a result, our studies confirm the beneficial effect of vanadium in experimental diabetes and suggest that this effect is due to its antioxidant properties.

The role of copper(II) ions in oxidative stress induced by glycation of human serum albumin with methylglyoxal

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Methylglyoxal (MGO), a reactive α-oxoaldehyde, is generated endogenously in higher levels in diabetes and causes carbonyl stress by reacting with proteins, leading to formation of advanced glycation end products (AGEs). Human serum albumin (HSA) is important source of antioxidants in circulation having its free Cys34 thiol group and a transporter of various ligands, including copper(II) ions, whose levels are reported to be increased in diabetes. Unbound copper(II) ions may undergo Fenton reaction causing oxidative stress by formation of free radicals. The aim of this study was to examine the affinity of MGO modified HSA to bind copper(II) ions.

HSA copper(II) complexes were incubated with MGO for 24 h, and HSA-MGO modified molecules were incubated with
basal glucose, HbA1c, triglycerides, total cholesterol, HDL-
measured and the blood concentrations of fasting glucose, post-
before and after the supplementation, body mass index (BMI),
None of the patients had any of the diabetic complications.
were administered for 2 months daily three capsules of
ory PUFA, each capsule containing 221 mg EPA and 147 mg DHA.
and PUFA. In type 2 diabetic patients the effects of daily intake of
n LC PUFA on circulating profiles of adipokines such as adipo-
be investigated as a preventive in diabetes treatment.
SW04.S16–64
Effects of n-3 Long chain PUFA on circulatory levels of adiponectin, IGF-1 and the proinflammatory cytokines, TNF-alpha, IL-1beta, IL-6 in type 2 diabetic patients
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n-3 Long chain polyunsaturated fatty acids (n-3 LC PUFA) represent healthy constituents of diets for patients with chronic inflammatory conditions including type2 diabetes mellitus. Adipose tissue is specifically linked to the beneficial effects of n-3 LC PUFA. In type 2 diabetic patients the effects of daily intake of n-3 LC PUFA on circulating profiles of adipokines such as adiponectin, IGF-1, TNF-α, IL-1β and IL-6 were investigated.

Thirty-nine type 2 diabetic patients, divided into two groups as receiving oral antidiabetic drugs (n = 27) and insulin (n = 12) were administered for 2 months daily three capsules of n-3 LC PUFA, each capsule containing 221 mg EPA and 147 mg DHA. None of the patients had any of the diabetic complications. Before and after the supplementation, body mass index (BMI), waist circumference and systolic/diastolic blood pressure were measured and the blood concentrations of fasting glucose, post-prandial glucose, HbA1c, triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol, adiponectin, IGF-1, TNF-α, IL-1β and IL-6 were determined.

Baseline concentrations of fasting and post-prandial glucose, HbA1c and IGF-1 were lower, but adiponectin higher in the oral antidiabetic group. At the end of the supplementation period, in both oral antidiabetic and insulin groups fasting glucose, HbA1c were reduced and adiponectin increased. Additionally in the oral antidiabetic group, TNF-α, total cholesterol, LDL-cholesterol and waist circumference were also reduced.

n-3 LC PUFA induced changes in adiponectin and fasting glucose was negatively correlated in the oral antidiabetic group whereas the changes in TNF-α and both fasting and post-prandial glucose were positively correlated in the insulin group. In both oral antidiabetic and insulin groups the changes in IGF-1 and interleukins and also the changes in TNF-α and IL-1β were positively correlated. In the oral antidiabetic group, the changes in diastolic blood pressure and BMI were positively correlated.

We have shown that supplementing type 2 diabetic patients with n-3 LC PUFA can increase the insulin sensitizing and anti-inflammatory adiponectin, whereas decrease the insulin resistant, proinflammatory cytokine TNF-α. Accordingly, an improvement was achieved in glycemic control.

SW04.S16–65
Efficacy of antioxidant vitamins (vitamin C, vitamin E, beta-carotene) and selenium supplement on d-galactosamine-induced lung injury in rats
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This study was designed to examine protective roles of the antioxidant vitamin supplementation combined with selenium against acute lung injury in rats. Sprague Dawley female rats were divided into four groups as follows: (I) rats injected physiological saline solution, intraperitoneally (ip), (II) animals treated with the combination of vitamin C (100 mg/kg/day), vitamin E (100 mg/kg/day), beta-carotene (15 mg/kg/day), and sodium selenate (0.2 mg/kg/day) for 3 days via gavage, (III) rats injected D-galactosamine (D-GalN; 500 mg/kg; ip) as a single dose, and (IV) rats given the antioxidant combination for 3 days, then injected D-GalN. Rats were sacrificed 6 h after the injection in groups I and III, 7 h after the last administration in groups II and IV. Lungs were investigated under light microscopy and used for protein, thromboplastic activity and enzyme analysis. In rats treated with D-GalN, lungs were characterized by extensive edema in peripheral areas, mononuclear cell infiltrations around venules and locally a honeycomb-like structure. Lung peroxoanase, Na+/K+ ATPase and glutathione peroxidase activities were decreased, while xanthine oxidase and thromboplastic activities were increased in rats treated with D-GalN. Pretreatments of the antioxidant vitamins combined with selenium preserved lung against injury by improving biochemical alterations and pulmonary edema in D-GalN-treated rats, whereas they have not had an effect on prevention of pulmonary inflammation in these rats. In conclusion, the antioxidant vitamins combined with selenium can be used in the prevention of acute lung injury.

SW04.S16–66
Runx2 gene plays a protective role in ureteral obstruction-induced kidney fibrosis through inhibition of TGF-β signal
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Runt-related transcription factor 2 (Runx2) plays an important role in bone formation and de novo synthesis of proteins, including type 1 collagen, matrix metalloproteinase 13, and fibronectin. Runx2 has a potent effect on signaling of transforming growth factor (TGF)-β and vice versa, implicating its significant role in fibrosis. Chronic renal failure comprises fibrosis, characterized as an increase in TGF-β signaling, and expression of α-smooth muscle actin (α-SMA), and extracellular matrix proteins, including collagens and fibronectin. In this study, we evaluated the role of Runx2 in ureteral obstruction (UO)-induced kidney fibrosis using mice whose Runx2 gene expression is genetically down-regulated. UO caused tubular atrophy and dilation, expansion of interstitium, and increased expression of collagens and α-SMA with a concomitant decrease in expression of Runx2. Deficiency of the
Runx2 gene (Runx2\(^{-/-}\) mice) showed higher expression of collagen and α-SMA, and histological damage in the kidney following UO, indicating exacerbated fibrosis, compared to wild type (Runx2\(^{+/+}\) mice). UO-induced activation of TGF-β and Smad3 was higher in the Runx2\(^{-/-}\) kidney than in the Runx2\(^{+/+}\) kidney, suggesting an inhibitory effect of Runx2 on TGF-β signaling in kidney fibrosis. In addition, overexpression of the Runx2 gene using an adenoviral vector in kidney tubule cells resulted in attenuated TGF-β-induced phosphorylation of Smad3 and expression of α-SMA and collagen I indicating inhibited transdifferentiation of kidney tubule cells into myofibroblasts. Furthermore, Runx2 gene deficient mouse embryonic fibroblasts induced greater activation of Smad3 and expression of α-SMA in response to TGF-β. Collectively, Runx2 plays a protective role in UO-induced kidney fibrosis by inhibition of TGF-β signal activation, suggesting Runx2 as a novel target for protection against fibrosis-related diseases such as chronic renal failure.

SW04.S16–67
Effect of bucillamine on hyaluronan degradation induced in vitro by reactive oxygen species

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High-molar-mass hyaluronan (HA) is a unique glycosaminoglycan present in many tissues of vertebrates. Under pathological conditions high-molar-mass HA often degrades yielding low-molar-mass HA fragments. Reactive oxygen species may initiate degradation of high-molar-mass HA often leading to a loss of its physiological function.

The present study was designed to test protective effect of antirheumatic drug bucillamine (BUC) against the free-radical-mediated HA degradation initiated by Weissberger’s biogenic oxidative system (WBOS), comprising 100-μM ascorbate plus 1-μM Cu(II). BUC was tested in 1–100 μM concentration range. Time-dependent decrease of HA dynamic viscosity reflecting the HA degradation was recorded by rotational viscometry. Oxymetry was applied to follow the overall process. Free-radical scavenging activity of BUC was evaluated by the ABTS and DPPH assays. HA fragments were studied by non-isothermal chemiluminesimetry (CL), Fourier-transform infrared spectroscopy (FT-IR), inductively coupled plasma mass spectrometry (ICP-MS) and size-exclusion chromatography with a multi-angle light scattering photometer (SEC-MALS).

Rotational viscometry revealed that BUC completely inhibited the WBOS-induced decrease of the dynamic viscosity of HA solution, which might indicate BUC-mediated protection of HA from its oxidative degradation. Also, scavenging of ABTS \(^+\) and DPPH by BUC (IC\(_{50}\) 4.00 and 8.96 μM, respectively) suggested its high radical scavenging activity, and oxymetry showed that BUC effectively reduced oxygen consumption after the initiation of the oxidative HA degradation. In contrast, the data of SEC-MALS indicated that the oxidative HA degradation took place also in the presence of BUC. Furthermore, CL demonstrated that BUC did not significantly prevent the WBOS-induced decrease of hydroperoxide content in the HA sample. Finally, FT-IR and ICP-MS demonstrated that generated BUC-thyl radicals did not incorporate into the HA biopolymer. Concluding, our data suggest that effects of BUC towards the oxidative HA degradation observed in vitro could be partially involved in preventing/treating the oxidative degradation of HA in vivo, though mechanism(s) of BUC action are not fully clear yet.

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SW04.S16–68
Tissue factor activities of kidney in D-galactose induced rat aging model

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Older age has been characterized as a ‘pro-inflammatory’ state and elevation of inflammatory and hemostasis markers has been implicated in many age-related chronic conditions. Animal and human experimental models suggest that older subjects have higher circulating levels of inflammatory and coagulation-fibrinolysis response. On the other hand decreased renal functions and increased susceptibility to age-related renal insufficiency are common symptoms of aging. D-Galactose is a hexose monosaccharide which is less soluble than glucose in water and D-galactose-induced aged rats have been shown to have significant similarities with the naturally aged rats in terms of impaired redox homeostasis and have been suggested to be used as a reliable animal model for renal ageing. Tissue Factor (also known as thromboplastin, TF) is the principal initiator of the coagulation cascade, and in addition to promoting blood coagulation, it is involved in inflammation, embryonic development, angiogenesis, tumour metastasis, cell adhesion/migration, and innate immunity. Various tissues and body fluids have been shown to have TF activity (TFa). The aim of this study is to investigate the changes in TFa of D-galactose induced rat renal tissues and compare with the TFa of the naturally aged and the control groups. Moreover sialic acid (SA) which is proposed as a valuable indicator for inflammatory diseases, as well as antioxidant markers are also determined in renal tissue samples. D-galactose (60 mg/kg/day) was injected intraperitoneally to young male (20 week old) Sprague-Dawley rats for 6 weeks. Naturally aging and control groups were injected with saline (0.9%). At the end of the treatment, the rats were sacrificed following an overnight fast, and the kidneys were immediately collected for homogenization. TF, Catalse, Superoxide Dismutase, Glutathione-S-transferase activities and glutathione, SA and malondialdehyde levels were evaluated in the homogenates, the results were evaluated statistically.

SW04.S16–69
Modeling of short-chain fatty acids metabolism in human gut: reconstruction of possible relations between microbiota composition and type 2 diabetes status

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The gut microbiota plays a key role in the process of human nutrition. It bridges metabolism of host tissues with indigestible nutrients such as complex sugars and glucans. In addition, it effects regulation of intestinal glucose absorption, lipogenesis and fat deposition. Recent metagenome studies highlighted role of the gut microbiota at dysbiosis, particularly, in abundance of butyrate pro-
Conclusions: A quantitative understanding of relationship between gut microbiota and physiologically relevant biomarkers can open new possibilities for the investigation of the risk factors, causing complex diseases, such as T2D. Form the other side such integrated approaches can be used for a development of the novel type of medicines targeting composition of gut microbial community.

SW04.S16–70
The role of lectin-like oxidized LDL receptor-1 as a mediator of endothelial dysfunction in patients with metabolic syndrome
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Background and aim: Metabolic syndrome (MetS) defines a well-known cluster of metabolic disturbances associated with an increased risk of cardiovascular disease and diabetes. In this study in patients with MetS and healthy controls the levels of soluble lectin-like oxidized LDL receptor-1 (sLOX-1), it’s possible association with oxidized LDL (oxLDL), endothelial nitric oxide synthase (eNOS), nitric oxide (NO), endothelin-1 (ET-1), paraoxonase 1 (PON1) and arylesterase (ARE) activities were examined.

Methods: 55 patients (37 women, 18 men) with MetS and 29 healthy controls (19 women, 10 men) with a body mass index (BMI) <25 kg/m² were enrolled in the study. sLOX-1, oxLDL, eNOS and ET-1 were determined by ELISA; NO, PON1 and ARE were measured by colorimetry.

Results: When compared to healthy controls the levels of sLOX-1, oxLDL, and ET-1 were significantly higher (p = 0.023, p < 0.001, and p < 0.001, respectively) but the levels of eNOS, NO, PON1 and ARE were significantly lower (p = 0.017, p < 0.004, p < 0.001, and p = 0.010, respectively) in patients with MetS. sLOX-1 was observed to be positively correlated with oxLDL, ET-1, BMI, glucose. ET-1 also exhibited significant negative correlation with ARE activity.

Conclusions: We have shown that in patients with MetS high sLOX-1 levels are associated with cardiovascular risk factors such as increased oxLDL, obesity and increased fasting blood glucose. An increased concentration of Lox-1 could be considered as an early predictor of endothelial damage in MetS. In addition, it appears that oxLDL, ET-1, eNOS, NO, PON1 and ARE may accurately reflect the levels of endothelial dysfunction in MetS patients.

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SW04.S16–71
Effects of melatonin receptor 1B gene variation on glucose control in population from Bosnia and Herzegovina
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Common variants in MTNR1B, encoding melatonin receptor 1B, have been recently associated with impaired glucose homeostasis and an increased risk for developing Type 2 diabetes (T2D). The aim of the study was to investigate the association of MTNR1B variant rs10830963 with T2D and related quantitative traits in a population from Bosnia and Herzegovina (BH). A total number of 268 subjects, 35–65 years old, were recruited in the study (162 T2D patients and 106 nondiabetic controls). Subjects were genotyped for MTNR1B rs10830963 SNP by using hydrolysis probes. Association of this genetic variant with T2D was analyzed by employing logistic regression model, while association with quantitative traits was assessed by using multiple linear regression. Both models were adjusted for age, gender, and body mass index. Our data showed that MTNR1B genotype frequencies in T2D and nondiabetic subjects were in accordance with Hardy-Weinberg equilibrium. The prevalence of the rs10830963 G allele in the population was 26%. We confirmed a significant association of MTNR1B rs10830963 with fasting plasma glucose levels in nondiabetic subjects. Under the additive genetic model, each variant G allele was associated with increased fasting glucose level of 0.29 mM (95% CI 0.12, 0.45, p = 0.001). Similar results were observed using dominant or recessive genetic model. We also showed a significant association of this MTNR1B polymorphism with increased glycated hemoglobin (Hb1Ac) in control subjects (p = 0.040, additive genetic model). An association of the MTNR1B variant rs10830963 with T2D risk and other related quantitative traits was not detected in our cohort. In conclusion, this is the first study replicating the association between the common MTNR1B gene variation and diabetes-related traits in BH population. We showed that MTNR1B rs10830963 polymorphism affected the fasting glucose and Hb1Ac levels, further strengthening the role of this variant in blood glucose control.
SW04.S16–72
Investigation of the tissue factor activities and antioxidant status of liver in D-galactose induced aging model
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Blood coagulation capacity increases with age in healthy individuals, apparently due to the increases in the plasma concentration of most procoagulant factors. This situation plays an important role in the advancing age-associated increase of cardiovascular diseases and thrombosis. Galactose is a structural component of membrane lipids and proteins in cells and D-galactose rat model has been used widely for aging research and drug testing since they have been shown to resemble their aged control counterparts of 16- to 24-months-old physiologically and pathologically. Accordingly increased production of free radicals, decreased antioxidant enzyme activity, and diminished immune responses have been shown in D-galactose-induced aging rat model. Tissue factor (TF) has been known as a key initiator of the coagulation cascade, and the TF pathway is the primary physiological mechanism of initiation of blood coagulation. Various tissues and body fluids have been shown to have TF activity (TFa). The aim of this study is to investigate the TF activities (TFa) of liver in D-galactose induced aging rats and compare with the natural aging and control groups. The groups were organized as follows: (i) young male control group (n = 8), (ii) naturally aged male group (n = 10), 3-D-Galactose induced aging model group (n = 10, 20 week old male rats, 60 mg/kg/day D-galactose injected for 6 weeks). Naturally aging and control groups were injected with saline (0.9%). At the end of the treatment, the rats were sacrificed following an overnight fast, and the liver tissues were immediately collected for homogenization. TF, Catalase, Superoxide Dismutase, Glutathione-S-transferase activities and glutathione, SA and malondialdehyde levels were evaluated in the homogenates, the results were evaluated statistically.

SW04.S16–73
Investigating the inadequate cellular stress response in peritoneal dialysis – a novel pathomechanism and its therapy
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Background: Peritoneal dialysis fluid (PDF) has cytotoxic effects on mesothelial cells (MC), which form an essential part of the peritoneal dialysis membrane. Depressed levels of heat shock proteins (HSP) upon exposure of cells to PDF represent a novel mode of cytotoxic action by an inadequate cellular stress response (ICSR). Our aim was to elucidate molecular mechanisms involved in ICSR and to test novel cytoprotective strategies.

Methods: To investigate the dynamic complexity of the PD-eﬄuent proteome, artificial PD-eﬄuents and clinical samples the PD-pecrote phase I trial (ClinicalTrials.gov; NCT01353638) were analyzed by gel-based fluorescent detection of protein abundance and MALDI-MS protein identification before and after deple-
Abstracts

**SW04.S16–75**

**Searching for pharmacological chaperones aiding to stabilize hydroxymethylbilane synthase**

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Mutations in the third enzyme of the haem biosynthesis, hydroxymethylbilane synthase (HMBS), give rise to the autosomal dominantly inherited disease acute intermittent porphyria (AIP). Diminished activity may cause acute attacks recognized mainly as abdominal pain and nausea. A need for better treatment is desirable to increase the quality of life for AIP-suffering patients.

Our aim was to search for pharmacological chaperones that may stabilise wild-type (WT) and HMBS mutants. Mutations affecting the activity due to changes in stability are the most likely candidates for pharmacological chaperone treatment.

Screening of compounds stabilising WT-HMBS was performed using a diversity library consisting of 10 000 compounds, and the thermostability of WT with compounds was analysed by differential scanning fluorimetry using a fluorescent dye. Sixty-four compounds were found to stabilise the enzyme ≥1.5°C. Of these, 40 were excluded due to the probability of being false hits based on knowledge attained with other proteins using the same library, and filters against pan assay interference compounds (PAINS). Structural analyses revealed few similarities between the 24 remaining compounds. These were further analysed for compound concentration stabilisation dependency, and 18 were selected for in vitro activity studies.

Preliminary activity measurements showed that the first nine compounds had no or weak inhibiting effect at standard conditions (37°C). However, weak inhibitors of other enzymes have been shown to be good pharmacological chaperones in vivo, and it is therefore important to investigate the effect of the compounds in cells expressing HMBS. Based on our previous studies, several mutants were found to be candidates for pharmacological chaperone treatment. In addition, as AIP is autosomal dominant, increasing the WT activity will also be of value.

**SW04.S16–76**

**HLA-B27 allele frequency in a Turkish population with primer osteoarthritis**

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**Aim:** Human Leukocyte Antigen (HLA) B27 is a class I surface antigen encoded by the B locus in the major histocompatibility complex (MHC) on chromosome 6 and presents antigenic peptides to T cells. It has been associated with arthritis diseases like Ankylosing spondylitis and Reiter syndrome. Osteoarthritis (OA) is the most prevalent arthritis disease worldwide. It is generally agreed that causation is multifactorial, involving age, gender, acute and chronic joint trauma, dietary factors, genetic predetermination and metabolic and inflammatory mechanisms. In literature, a study explaining the frequency of HLA-B27 allele in OA has not been come across. Thus in this study we aimed to determine the frequency of HLA-B27 allele in patients with primer OA in a Turkish study population.

**Materials and Methods:** Genomic DNA was extracted from 87 patients with primer OA and 51 healthy controls in the study. DNA was amplified by using HLA-B27 PCR kit. Products were assessed with UV transilumator by being exposed to 2% agarose gel electrophoresis.

**Results:** Allele frequencies between groups were compared using the chi-square test. There was no significant difference between patients and controls. Frequency of HLA-B27 was %16 in patients and %19 in control group.

**Conclusion:** This study gives an idea about the frequency of HLA-B27 in primer OA. We may assert that the frequency of HLA-B27 in primer OA does not differ from the healthy population. The frequencies we found in both groups are also in consistent with the frequency which is found 9% in Caucasian worldwide for HLA-B27. However the significance of our result remains to be further investigated in different and even larger populations.

**SW04.S16–77**

**Alterations of thyroid hormone levels in cadmium exposure**

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**Objective:** Environmental chemicals and heavy metals might alter thyroid hormone levels via several mechanisms, including disruption of iodine (I) transport, thyroid peroxidase, thyroid hormone-binding proteins, hepatic catabolism, deiodinases, and receptor binding. Our aim was to investigate the thyroid hormone levels in cadmium exposure.

**Materials and Methods:** 1724 male participants aged between 18 and 72 years were included in this study and divided into six groups according to whole blood cadmium levels (Group 1: 0–0.5 μg/l; Group 2: 0.5–1 μg/l; Group 3: 1–1.5 μg/l; Group 4: 1.5–2 μg/l; Group 5: 2–2.5 μg/l; Group 6: >2.5 μg/l). Whole blood cadmium levels were determined by Ankara Occupational Diseases Hospital toxicology laboratory using Varian AA 240Z atomic absorption spectrophotometry. Serum thyroid hormones (free triiodothyronine, free thyroxine and thyroid stimulating hormone) and vitamin B12, folic acid, Aspartate transaminase, Alanin transaminase, Urea analyses were performed in Roche Cobas 6000 E601 autoanalyzer.

**Results:** There was no significant difference for serum thyroid stimulating hormone, Aspartate transaminase and folic acid levels between six groups (p = 0.187, p = 0.193 and p = 0.467, respectively). While serum Vitamin B12 and urea levels were higher in Group 1 compared to other groups, serum Alanin transaminase levels were found to be higher in Group 1. Serum free triiodothyronine and free thyroxine levels were significantly lower in Group 1 compared to other groups (p < 0.001). There was a positive correlation between cadmium and serum free thyroxine, triiodothyronine and urea levels (r = 0.167, p < 0.001;
SW04.S16–79
Thrombin binds human ceruloplasmin and proteolytically hinders its antioxidant activity: implications in the pathogenesis of rheumatoid arthritis

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Human ceruloplasmin (CP) is a member of the multi-copper oxidase family and plays a key role in iron metabolism. Purified CP preparations contain the intact protein (132 kDa) and, depending on the sample, variable amounts of nicked species are produced by some yet unidentified protease.

In this study we have demonstrated that thrombin, a key protease in coagulation, inflammation and cell proliferation, cleaves CP both in vitro and in the synovial fluid of patients (24) with rheumatoid arthritis (RA) at Arg481-Ser482 and Lys887-Val888 peptide bonds, thus generating a nicked species (CP*) formed by a non-covalent complex of fragments 1–481, 482–887, and 888–1046. Strikingly, intact CP inhibits MPO and functions as an antioxidant both in vitro and in vivo; conversely, CP*, while retaining the ferroxidase activity of CP, has lost the MPO inhibitory function of the intact protein. Remarkably, the concentration of thrombin, MPO and CP* are increased in the synovial fluid of RA patients. Moreover, active-site blocked thrombin competitively inhibits the ferroxidase function of CP (Ki = 0.2 μM). Direct analysis of thrombin-CP interaction was carried out by surface plasmon resonance (SPR) and fluorescence spectroscopy, using the inactive thrombin mutant S195A, yielding a Kd of 2.0 and 0.4 μM, respectively. Displacement SPR experiments, carried out with specific binders of thrombin exosite-1 (hirugen and HD1 aptamer) or exosite-2 (fibrinogen gamma'-peptide and HD22 aptamer), indicate that both exosites are involved in CP binding. Interestingly, cleavage of CP is abolished in the presence of hirudin, a potent and selective thrombin inhibitor, which also reduces joint inflammation in RA patients.

In conclusion, we have shown here that thrombin cleaves CP and proteolytically hinders the inhibitory effect that intact CP exerts on MPO function. These results are unprecedented and set the basis for elucidating the biochemical mechanism underlying the progression of inflammation in RA patients.

SW04.S16–80
Molecular mechanism of body weight reducing effect of oral boric acid intake

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Objective: We have previously showed the effect of oral boric acid intake on reducing body weight in a mouse model (1). In order to reveal molecular mechanism of body weight reducing effect for oral boric acid intake, gene expression levels for uncoupling proteins (UCPs) was measured in different tissues isolated from boric acid fed mice.

Methods: Twelve mice were used, in groups of six each in the control and study groups. For 5 days, control group mice drank standard tap water while during the same time period the study...
group mice drank tap water which contains 0.28 mg/250 ml boric acid. After a 5 day period, gene expression levels for UCP1, UCP2, and UCP3 in the white adipose tissue (WAT), brown adipose tissue (BAT) and skeletal muscle tissue (SMT) and total body weight changes were analyzed.

**Results:** Real time PCR analysis revealed no significant change in UCP3 expressions but UCP2 in WAT (p: 0.0317), BAT (p: 0.014), and SMT (p: 0.0159) and UCP1 in BAT (p: 0.026) were overexpressed in the boric acid group. In addition, mice in the boric acid group lost body weight (mean 28.1%) while mice in the control group experienced no weight loss but, a slight weight gain (mean 0.09%, p < 0.001).

**Conclusion:** Oral boric acid intake causes overexpression of thermogenic proteins in the adipose and skeletal muscle tissues. Increasing thermogenesis through UCP protein pathway results in the accelerated lipolysis and body weight loss.

**Reference**

**SW04.S16–81**
**Sodium tungstate decreases the progression of renal damage through inhibition of fibrosis in diabetic rat kidney**

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Diabetes mellitus is a condition in which the metabolism of carbohydrates, lipids and proteins are inadequately regulated by insulin. One of their complications is diabetic nephropathy, where pathological accumulation of extracellular matrix proteins and renal fibrosis are the fundamental mechanisms by which this disease progresses to end-stage renal failure. In this study, we evaluate the progression of renal damage in long term streptozotocin-induced diabetic rats through anatomopathological and biochemical analysis and we evaluated the effect of sodium tungstate (NaW) on the diabetic nephropathy. The anatomopathological analysis showed glomerular damage since 4 months of diabetes and increases constantly to 8 months. At the final stage we found damage in glomeruli by observing increased glomerular sclerosis (16%). Moreover, tubular damage is the first to appear beginning in the second month of induction of diabetes. Surprisingly, sodium tungstate reduced the progression of nephropathy on early and long-treated diabetic rats. In addition, we evaluated the expression of several markers of fibrosis in the progression of this pathology. We observe that the increase in the protein levels of type IV-collagen and α-SMA produced by renal damage were reverted by treatment with sodium tungstate. Our results indicate that damage progression in diabetic nephropathy is guided by a process involving fibrosis and sodium tungstate treatment is able to reverse this process and slow down the progression of kidney damage (CONICYT 2110230).

**SW04.S16–82**
**Sodium tungstate attenuates fibrosis through suppression of transforming growth factor-β1/Smad3 in diabetic nephropathy**

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Diabetes mellitus induces significant complications in human patients, among these diabetic nephropathy (DN). Sodium tungstate (Na2WO4) has been described as a potent normoglicemician-

**SW04.S16–83**
**Post-translational modulation of hydrogen peroxidation enzymes by streptozotocin induced diabetes and antioxidants**

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Present study investigated the changes in gene and protein expressions as well as catalytic activities of catalase and glutathione peroxidase by diabetes and antioxidants. Total protein, catalase and glutathione peroxidase phosphorylation levels were also determined. According to results; phosphoprotein profiles of diabetic and antioxidant treated rats were modified and total protein phosphorylation was increased by diabetes and vitamin C. As parallel with mRNA, total catalase protein amount and activity were also decreased in diabetes and both antioxidants elevated this reduction towards the control levels. Unlike total catalase, phospho catalase levels were further suppressed by diabetes and antioxidant treatments. Moreover, diabetes and antioxidants did not change the unphospho and phospho glutathione peroxidase protein levels. Findings revealed that suppression of catalase activity in diabetes were at the level of gene expression and its activity was further modulated by a post-translational mechanism mostly via phosphorylation with diabetes and antioxidants mainly with vitamin C.

**SW04.S16–84**
**Metabolic aspects of toxification and detoxication of the anticancer agent ellipticine determining its pharmacological efficiencies**

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The anti-tumor therapeutic ellipticine is a prodrug, whose pharmacological efficiency is dictated by its cytochrome P450 (CYP) and/or peroxidase-mediated activation in target tissues. The CYP1A1-mediated ellipticine metabolites 9-hydroxy- and 7-hydroxyellipticine and the product of ellipticine oxidation by peroxidases, the ellipticine dimer, are the ellipticine detoxication metabolites. In contrast, two carbonium ions, ellipticine-13-ylum and ellipticine-12-ylum, derived from two activation elliptici-
metabolites formed by CYP1A and 3A, 13-hydroxyellipticine and 12-hydroxylupeptine, generate two deoxyguanosine adducts in DNA found in the human breast adenocarcinoma MCF-7, leukemia HL-60 and CCRF-CEM, neuroblastoma UKF-NB-3 and UKF-NB-4, glioblastoma U87MG and thyroid cancer 8505-C, BCPAP a BHT-101 cells and in rat breast carcinoma in vivo. Formation of these covalent DNA adducts by ellipticine is the predominant mechanism of its cytotoxicity and anti-tumor activity to these cancer cells. Recently, we have found that enzymatic activities of CYP1A and 3A are modulated by cytochrome b5; whereas the amounts of the detoxication ellipticine metabolites are either decreased or not changed with cytochrome b5 added to the reconstituted CYP1A1/2 and 3A4 systems, the amounts of the activation metabolites, 12-hydroxy- and 13-hydroxylupeptine, increased considerably. Ellipticine is also an inducer of CYP1A, 1B1 and 3A4 enzymes and cytochrome b5 in the cancer cells and in vivo in rats exposed to this compound, thereby modulating its own pharmacological efficiencies. Our results form the basis to further predict the susceptibility of human cancers to ellipticine and suggest this prodrug for treatment in combination with CYP and/or peroxidase gene transfer increasing its anticancer potential. It also suggests ellipticine reactive metabolites 13-hydroxylupeptine and 12-hydroxylupeptine to be suitable candidates for targeting to tumors absenting the CYP and peroxidase activation enzymes.

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**SW04.S16–85**

**Impact of TP53 status on the metabolic activation of environmental carcinogens in human cells**

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More than 50% of human tumours contain a mutation in the TP53 gene and exposure to several environmental carcinogens has been linked to characteristic mutations in TP53. The Hupki (Human TP53 knock-in) mouse embryo fibroblast (HUF) immortalisation assay (HIMA) is an important model for studying the impact of environmental carcinogen exposure, for example benz[a]pyrene, on TP53 mutagenesis. In addition, we found that the cellular TP53 status can influence the metabolism of different environmental carcinogens, suggesting a potential role of TP53 in the regulation of xenobiotic-metabolising enzymes (XMEs). To further investigate the role of TP53 in the cytochrome P450 (CYP)-mediated metabolism of several polycyclic aromatic hydrocarbons (PAHs) - benz[a]pyrene, dibenzo[a,l]anthracene and dibenzo[a,l]pyrene - a panel of isogenic colorectal HCT116 cells were used, differing only with respect to their TP53 status. HCT116 cells having TP53(+/+), TP53(−/−), TP53(−/+), TP53(R248W−/−) or TP53(+/+R248W) were treated with 2.5 μM of the PAH for 24 or 48 h. Parent PAHs resulted in significantly higher DNA adduct levels in TP53(+/+) cells measured by 32P-postlabelling compared to the other cell lines whereas exposure to the corresponding activated PAH diol-epoxides induced similar adduct levels in all cell lines. Western blot analysis showed that CYP1A1 protein expression was induced after PAH treatment to much greater extent in TP53(+/+) cells compared to the other cell lines, whereas AHR expression decreased relative to controls in all cells to the same extent, independent of TP53 status. Since PAH diol-epoxides, unlike parent PAHs, do not require metabolic activation to form DNA adducts these results clearly show that the cellular TP53 status is linked to the CYP1A1-mediated bioactivation of the PAHs tested. Investigations are underway to determine whether these phenomena are observed with other classes of environmental carcinogens.

**SW04.S16–86**

**Metabolism of the human carcinogen aristolochic acid**

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The use of traditional herbal medicines is increasing worldwide. The old herbal drug aristolochic acid (AA), found in the *Aristolochia* genus of plants has been associated with the development of a novel nephropathy, designated as aristolochic acid nephropathy (AAN). It also became apparent that damage due to AA can persist for several years and that AA is associated with an extremely high incidence of urothelial cancer. The principal components of the plant extract are nitrophenanthrene carboxylic acids, which are genotoxic mutagens after metabolic activation. The major activation pathway involves reduction of the nitro group primarily catalysed by NAD(P)H:quinone oxidoreductase to an electro-philic cyclic N-arylnitrenium with delocalised charge that reacts preferentially with purine bases to form covalent DNA adducts. These aristolochic acid specific DNA adducts have been identified and detected in experimental animals exposed to aristolochic acid or botanical products containing aristolochic acid, and in renal tissues from AAN patients. In rodent tumors the major adduct formed by AA has been associated with the activation of ras oncogenes through a specific A:T to T:A transversion mutation in codon 61. A:T to T:A transversions were also the predominant mutation type found in human p53 knock-in mouse fibroblasts treated with AA. In humans A:T to T:A transversions in the p53 gene have been identified in several patients suffering from Balkan endemic nephropathy (BEN) and in a urothelial tumor from an AAN patient along with AA-specific DNA adducts. This concordance of specific mutations in patient tumours and AA-exposed cells supports the argument that AA was responsible for the high risk for cancer in individuals who ingested material from *Aristolochia* plants in the form of weight-loss pills in Belgium, or from cereal fields in the Balkans where *Aristolochia clematitis* grow as weeds. IARC has classified AA as carcinogenic to humans (Group 1) and has urged a ban of all botanical products known or suspected to contain AA from the market worldwide. Despite this ban urothelial carcinoma attributable to the use of *Aristolochia* plants seems to be more common than previously thought.

**SW04.S16–87**

**Anticancer potential of microbial RNases**

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Some ribonucleases (RNases) possess preferential cytotoxicity against malignant cells. The best known of these RNases, onconase, was isolated from frog oocytes and is in clinical trials as an anticancer drug. We turned our attention to *Bacillus intermedius* RNase (binase) as a potential agent for anticancer therapy. Binase possesses cytotoxic and cytostatic activity against malignant cells comparable to the activity of eukaryotic cytotoxic RNases; it has low immunogenicity and appears to have acceptable safety profile. We have shown that binase induces selective apoptosis of fibroblasts transduced by ras oncogene and myeloid progenitor cells FDC-P1 transduced by activated KIT oncogene; oncogenic KIT transcript is a target for binase. Binase reduces the amount of RNA in sensitive cells, but this decrease by itself is not fatal, it is the disruption of RNA-dependent regulatory processes that causes cell death.
Numerous cytogenetic abnormalities associated with acute myelogenous leukemia (AML) involve the genes encoding AML1-ETO, KIT and FLT3. Expression of both KIT and AML1-ETO oncogenes makes FDC-P1 cells sensitive to the toxic effects of binase. Kasumi-1 cells, expressing both of these oncogenes, were the most responsive to the toxic actions of binase. Either blocking the functional activity of the KIT protein with imatinib or knocking-down oncogene expression using lentiviral vectors producing shRNA against AML1-ETO or KIT eliminated the sensitivity of Kasumi-1 cells to binase toxic action and promoted their survival. Our data suggest that the cooperative effect of the expression of mutated KIT and AML1-ETO oncogenes is crucial for selective toxic action of binase on malignant cells. In addition we show that BAF3/FLT3-ITD cells are much more sensitive to the binase cytotoxic effects than the original BAF3 cells. Increased binase cytotoxicity toward the cells, expressing FLT3-ITD oncogene, suggests that, as in the case of FDC-P1 cells transduced by KIT oncogene the expression of an activated oncogene determines the sensitivity of cells to binase. These findings can facilitate clinical applications of binase providing a useful screen based on the presence of the corresponding target oncogenes in malignant cells.

**SW04.S16–88**

**New insight into regulatory mechanisms of CoA biosynthesis**

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Coenzyme A (CoA) is a fundamental cofactor in all living organisms. CoA and its derivatives are key players in diverse metabolic reactions, signalling pathways and regulation of gene expression. Deregulation of CoA biosynthesis and homeostasis has been observed in human pathologies, such as diabetes, neurodegeneration and cancer. Therefore, tight regulation of CoA and its derivatives is fundamental to ensure cell’s homeostasis. CoA Synthase (CoASy) mediates two final stages of de novo CoA biosynthesis. To date, very little is known about regulation of this important metabolic enzyme in response to various extracellular stimuli. We were the first to report molecular cloning and characterization of mammalian CoASy. We have subsequently showed that CoASy is localized on outer mitochondrial membrane, where it forms regulatory complexes with signaling molecules in particular with ribosomal protein S6 kinase, suggesting a functional link with protein biosynthetic pathways. Bioinformatic analysis of CoASy revealed the presence of motifs which can mediate its binding to signaling molecules, including p53 regulatory subunit of PI3K (p53pPI3K) and tyrosine phosphatase Shp2PTP. We have reported that CoASy forms a functional complex with p53pPI3K in a growth factor dependent manner. Significant changes in PI3K signaling were observed by siRNA-mediated CoASy knockdown, pointing on the role of CoA biosynthetic pathway in signal transduction. Taken together these studies uncover a potential link between the PI3K/mTOR/S6K signaling and energy metabolism through CoA and its derivatives. A function interaction between CoASy and Shp2PTP has been detected in vivo. We showed that endogenous CoASy is phosphorylated on tyrosine and Shp2PTP-mediated CoASy dephosphorylation leads to an increase in CoASy enzymatic activity. We therefore argue that regulation of CoASy phosphorylation represents unrecognized before mechanism of modulation intracellular CoA level in response to extracellular stimuli. Further identification of EDC4 protein, known as a central scaffold component of processing bodies, as a novel binding partner of CoASy represents a possible mechanism of negative regulation of CoA biosynthesis since EDC4 strongly inhibits CoASy dephospho-CoA kinase activity in vitro.

**SW04.S16–89**

**DNA polymerase beta and the BER pathway: toward a new approach to anti-cancer drug design**

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Base excision repair (BER) is a DNA repair mechanism that is believed to play a role in determining the sensitivity of cancer cells to DNA-damaging therapeutic agents. A key protein implicated in BER is DNA polymerase β (pol β). When over-expressed in cancer cells, pol β has been previously shown to reduce the effectiveness of several anti-cancer agents, and is also prone to mutations that increase its error rate in a significant fraction of tumors. As a result, it has been recognized as a potential target for novel anticancer drugs. A challenge in realizing the potential of this new approach for cancer chemotherapy has been to identify inhibitors that are both highly potent and highly selective for pol β, a goal that has not been achieved as yet using chemical library screening methods. Recently, our laboratory synthesized as mechanistic probes for pol β the first examples of individual stereoisomeric β−γ-CHX analogues of dGTP where X = F or Cl and showed that they can have discrete 31P or 19F NMR spectra, facilitating analysis under turnover conditions. These compounds, which are diastereomers distinguished only by the configuration of the bridging CXY carbon and thus by the position in space of the substituting halogen atom, exhibit stereospecificity, manifested as different Kᵢ and kᵣ values with pol β. When bound in a ternary complex of the enzyme and DNA, the preferred fluoro-analogue has its F atom 3 A from a guanidinium nitrogen of Arg183 in the active site. The implications of these and other recent results for realizing the goal of specific pol β inhibition using ligand design techniques based on the active site structure will be discussed.

**SW04.S16–90**

**Molecular function of the long noncoding RNA SPRY4-IT1 in human melanomas**


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Recently, we reported that the long noncoding RNA SPRY4-IT1, which lies within the intronic region of the Sprouty4 gene, is upregulated in human melanomas and influences cell growth, proliferation, and motility, suggesting it may play an important role in the molecular etiology of human melanomas. Here, we report the identification of the full-length of the SPRY4-IT1 transcript and its molecular function in human melanoma cells. The lenti-viral ectopic expression of SPRY4-IT1 in melanocytes induce the formation of multinucleated giant cells and modulate the expression of subsets of genes involved in apoptosis, cell cycle regulation, DNA packaging, chromosome organization, and chromatin architecture. In particular, SPRY4-IT1 expression results the downregulation of the tumor suppressor gene dipetidyl peptidase IV (DPPIV) and upregulation of the cellular proliferation marker Ki67. RNA immunoprecipitation followed by mass spectrometry analysis revealed that LIPIN2, but not LIPIN1
is binding to SPRY4-IT1. Interestingly, when LIPIN2 is knock-down, SPRY4-IT1 expression is decreased, but in contrary, when SPRY4-IT1 is knockdown LIPIN2 protein was increased. These results demonstrate an existence of a novel regulatory mechanism between SPRY4-IT1 and LIPIN2. Northern blot analysis and RNA-FISH results reveal that the presence of two SPRY4-IT1 transcripts, where longer version resides in nucleus and smaller version is predominantly found in the cell cytoplasm and accumulate in the polysome fraction of the cell, suggesting that this transcript may interact with cellular proteins and/or mRNAs. All above results exhibit that SPRY4-IT1 may play a key role in the transition of melanocytes to melanomas.

**SW04.S16–91**

**Determination of kinetic parameters of telomerase inhibition by telomerase RNA template antagonist**

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Telomerase is a specialized reverse transcriptase responsible for maintaining the termini of linear chromosomes. The human enzyme is a ribonucleoprotein complex minimally comprising a catalytic protein moiety (hTERT) and an RNA subunit (hTR) which acts as the template for the reverse transcriptase reaction. Inhibiting or blocking the activity of telomerase may be an important approach to targeting cancer, that may be applicable in a tumor types with short telomere.

Nowadays, there was developed telomerase inhibitor – Imetelstat (GRN163L) which has complementary sequence to hTR and which in clinical trial. Our goal was measuring the kinetic parameters of inhibition of telomerase by telomerase template antagonist analog of GRN163. In contrast to GRN163 which has i′ phosphoroamidate modification in backbone, we used meG-RN163 which has same sequence but has 2′OMe modification. We found that oligonucleotides with telomere sequences inhibit the PCR of RQ-TRAP. Though, we improved RQ-TRAP by adding dilution step before PCR. But that step greatly reduces the signal. To overcome that problem, we improved system by the over-expression of telomerase components (by enhancing the signal). To overcome that problem, we improved system by the over-expression of telomerase components (by enhancing the signal). To overcome that problem, we improved system by the over-expression of telomerase components (by enhancing the signal).

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According to GPC data, both Mlph-DOG-L and MTX-DOG-L have considerable amount of protein (~80 g protein/mol lipids). However, repertoires of bound proteins differed. While C4-binding protein of the complement and apolipoprotein E were detected among proteins associated with both liposome species, only MTX-DOG-L bound component C3 and complement regulatory factor H. The data correlate well with the increased complement consumption by MTX-DOG-L, and not Mlph-DOG-L, revealed previously [Kuznetsova et al, J Control Release, 2012]. Decrease in MTX-DOG content in the bilayer (10–2.5 mol%) resulted in the absence of C3 or factor H from the liposome surface and lack of unwanted complement activation under the experimental conditions and thus resolved the issue.

The work was supported by the Russian Foundation for Basic Research (project no. 12-04-31739).

**SW04.S16–93**

**Antitumor liposomes loaded with lipophilic prodrugs**

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To improve pharmacological properties of chemotherapeutical medicines, it is suggested to include them in the form of lipophilic prodrugs (LPS, ester drug-lipid conjugates) in the lipid bilayer of 100-nm-liposomes. Liposome preparations on the basis of egg phosphatidylethanolamine and phosphatidylserine from S. cerevisae bearing LPS of well-known antitumor remedies such as methotrexate and melphan (L-sarcolysine), as well as LPS of new antivascular agent for anticancer therapy combretastatin A-4 were designed. Standard extrusion of the mixture of all components through calibrated nuclear filters at 4°C yields liposomes with loading capacity 10–15 mol.% of LPS. The dispersions contain relevant concentrations of LPS to be effective in the therapy by systemic injections to animals. It was shown in vitro that LPS in liposomal formulations are stable to the premature hydrolysis by chemical cleavage.
human plasma esterases. To achieve targeting to the angiogenic tumor endothelium, liposomes are equipped with tetrasaccharide selectin ligand SiaLeX³. Selectins are expressed on the activated leucocytes, platelets, and endothelium cells. In the mouse models of mammary cancer, and lymphoma/leukemia liposomal formulations of the LPs inhibited tumor growth and prolonged the survival more efficiently than the intact drugs, the effect being particular strong when SiaLeX³-conjugated was included in liposomes. On the model of Lewis lung carcinoma, SiaLeX³-liposomes loaded with LP of melphanal were shown to provoke expressed antivascular and/or antiangiogenic antitumor effect. Also, they revealed moderate inhibition of metastasis formation. In the in vitro tests liposomes loaded with LPs of melphanal and methotrexate, including SiaLeX³-liposomes were shown to be hemocompatible. Moreover, employment of phosphatidylinositol for the stabilization of liposomes in circulation permits to avoid side effects related to immunogenic and toxic properties of polyethylene glycol conjugated phospholipids.

**SW04.S16–94**

**Novel approaches to combat cancer**

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**Background:** Curcumin (Cm), a polyphenol present in the spice turmeric (Curcuma longa) has anti-oxidant, anti-inflammatory, and anti-tumoral effects, but its use in therapy is limited because of the poor solubility. We hypothesize that encapsulation of curcumin into nanoparticles could overcome this problem. The aim of this study was to develop curcumin-loaded lipid nanoparticles able to treat atherosclerosis and cancer.

**Observations:** Cm-loaded nanoparticles (CmLN) have been developed using FDA-approved lipids and polymers. Phosphatidylcholine, polyethylene glycol and Cm were dissolved in soybean oil and sonicated in an aqueous phase. The obtained CmLN were characterized for size and structure (electronic and atomic force microscopy), entrapment efficiency, cytotoxicity and in vitro release of Cm. We also studied the endocytosis pathways used by CmLN in human vascular endothelial cells, breast and prostate cancer cells. For the treatment of atherosclerosis, we observed on vascular endothelial cells that low doses of CmLN have: (i) low cytotoxicity; (ii) high internalisation; (iii) anti-oxidant effect, decreasing the H₂O₂-induced reactive oxygen species; (iv) anti-inflammatory effects by down-regulating JNK, ERK1/2 and p38 MAPK signaling pathways; (v) impaired the adhesion of monocytes to activated endothelial cells. For cancer studies, two prostate (PC3) and breast (MCF-7) cancer cell lines have been employed. The studies revealed: (i) high internalisation of CmLN in both cell lines; (ii) CmLN effectively suppress cancer cells proliferation; (iii) the anti-proliferative effect of CmLN is dose-dependent and is higher compared to free curcumin.

**Conclusions:** The results of this study have revealed the dual role curcumin in treating two different pathologies. At lower doses curcumin is not toxic and has anti-inflammatory effects, beneficial for many inflammatory-driven pathologies, like atherosclerosis. By increasing the dose, curcumin can stop the proliferation of cancer cells. The poor solubility of curcumin has been overcome in our study by encapsulation in lipid nanoparticles, which provides a time-dependent release of Cm and protect its degradation.
SW04 Molecular Mechanisms of Disease

A new approach for targeted drug delivery into tumor cells


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The low efficacy of traditional antitumor chemotherapy is explained mainly by poor selectivity of antitumor drugs against cancer cells and the risk of multidrug resistance (MDR) development. Specially designed drug delivery systems are a good alternative to improve the therapeutic effectiveness and reduce the systemic toxicity of antitumor drugs. Vector molecules using for targeted delivery of antitumor drugs provides their binding selectivity on the surface of tumor cells and internalization into their endolysosomal compartment. The recombinant epidermal growth factor (EGF), EGF receptor binding peptides and receptor-binding fragment of alpha-fetoprotein (rAFP3D), were used as vector molecules. The EGFR and alpha-fetoprotein receptor are universal tumor markers, being expressed on the surface of many tumor cells. A three-component delivery system including vector protein, PAMAM G2 dendrimer (G2) and antitumor antibiotic doxorubicin (Dox) was synthesized. Two approaches of conjugation vector proteins to G2 were elaborated: (i) vector molecules were directly attached to G2 by using carbodiimide as well as Dox (via acid labile linker); (ii) vector molecules were attached to G2 through PEG-spacer, which was attached to the surface of G2 via acid labile linker (Dox was noncovalent incorporated into inner space between PEG arms). The attachment of dendrimer molecules to the vector protein did not affect the binding effectiveness to AFP and EGF receptors on the surface of tumor cells nor the effectiveness of receptor-mediated endocytosis. The in vitro Dox release study showed that the conjugate was stable at neutral pH but was labile at pH < 6. The Dox release was correlated with the intracellular distribution of conjugate in tumor cells. The conjugates showed a high cytotoxic activity against Dox-sensitive and Dox-resistant cell lines and was low-toxic against human peripheral blood lymphocytes. Based on our findings, we may conclude that it is possible to significantly increase the effectiveness of Dox delivery to tumor cells by using the targeted delivery system comprising G2 (as a drug carrier) and the rAFP3D, EGF or EGFR binding peptides (as a vector molecules).

Silver containing structures are being used widely and increasingly in various products and medical supplies due to their biological activity. However, little is known about impacts of stabilized-silver ion solution (St-Ag ion solution). The purpose of this study was to investigate the effect of St-Ag ion solution on cell viability, membrane and DNA damages on parental and drug resistant human lung cancer cells (H1299) after the cells treated with this solution. The results show that the St-Ag ion solution could be taken up by cells, decreased cell viability in dose-dependent manner at dosage levels between 0.5 and 4 µl. It caused increasing malondialdehyde level, an end product of lipid peroxidation and 8-OHdG formation, a product of oxidative DNA damages flowing specific enzymic cleavage after 8-hydroxilation of guanine base. The highest membrane and DNA damages were caused by St-Ag ion solution at 1C70 concentration in drug-resistant cell lines. Thus, our data suggest St-Ag ion solution has different potential toxicity on parental and drug resistant cancer cells depend on concentration that is associated with oxidative stress. Also, anticancer potential of anticancer drugs can be increased by combining therapy with stabilized-silver ion solution.

The potential of urinary volatile metabolites as a non-invasive, innovative and promising strategy for early diagnosis of cancer

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Despite of global efforts to limit the incident of this disease, cancer continues to be the major source of morbidity and the second most common cause of mortality worldwide. Breast and prostate cancer are respectively, the two forms of cancer most prevalent in women and men. Both have huge psychological, economic and social impacts in our societies and the current diagnostic tools present limited sensitivity and specificity. Specifically, mammography that is often used in the diagnostic of breast cancer is uncomfortable and not effective in the early stages of the disease and furthermore the exposure to radiation used is potentially hazardous. Hence, new and more efficient diagnostic approaches are necessary to invert this scenario. The management of these high-risk cancers requires diagnosis at an early stage, which specifies the need for more specific and sensitive biomarkers. Although various compounds from blood, saliva and urine have been explored as biomarkers, including proteins, tumor antigens, anti-tumor antibodies, cell type-specific peptides, metabolic and epigenetic products such as hyper-methylated DNA, RNA, and the expression of specific genes, most of them continue to fail in reaching clinical use. The cancer cells metabolism is necessarily different from their normal counterparts, being certain molecules differentially expressed in cancer cells. Many of these metabolites are volatiles and could be used to establish a cancer signature to differentiate health from disease. With the development of high-throughput techniques, the characterization of these volatiles using gas chromatography combined with mass spectrometry,
(GC-qMS) approach is feasible and will certain contribute to increase our knowledge about the cancer etiology and consequently improve the diagnostic tools that are available.

**SW04.S16–100**

**Cisplatin effects on nuclear neutral lipids of rat thymus cells**

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**Introduction:** The discovery of cisplatin, was a corner stone which triggered the interest in platinum(I)- and other metal-containing compounds as potential anticancer drugs. Clinical use of cisplatin determined that many patients with different types of cancer have been successfully treated. One of the first articles described a complete remission in a patient with renal metastases from thymoma who was treated with cisplatin. It is generally considered as a cytotoxic drug which kills cancer cells by damaging DNA and inhibiting DNA synthesis. However, only a small fraction of cisplatin actually interacts with DNA. Therefore, inhibition of DNA replication cannot solely account for its biological activity. Platinum compounds damage tumors via induction of apoptosis, which is mediated by the activation of various signal transduction pathways. Much attention has been paid to the interaction of cisplatin with DNA and -SH groups of proteins and peptides. However, there is only little information available on how cisplatin interacts with lipids which play an important role in the transduction of signals to the genome. Here cisplatin in vivo action on rat thymus cells nuclear neutral lipids has been studied.

**Methods:** The 24-h in vivo effect of cisplatin has been investigated. Rat thymus cells neutral lipids were fractionated by micro-TLC technique. The quantitative estimation of fractionated phospholipids was evaluated by using the computer program FUGIFILM Science Lab. 2001 Image Gauge V 4.0.

**Results:** Six individual fractions of neutral lipids were revealed in nuclei of rat thymus cells. Content of monoacylgllycerols and diacylglycerides were decreased by 23% and 34.5% respectively, while the content of cholesterol increased markedly (45%) after the cisplatin action. The quantities of triacylglycerides, cholesterol, cholesterol asters and free fatty acids remained almost unchanged.

**Conclusion:** These changes of content of individual fractions of neutral lipids in nuclei of rat thymus cells show the effect of cisplatin on nuclear lipid metabolism. More research is necessary to improve these changes in nuclear membranes and intranuclear structures.

**SW04.S16–101**

**In vitro effects of selenium on human glioblastoma multiforme cell lines**

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**Purpose:** The incidence of brain tumors ranges from 3 to 5 per 100 000 in humans. 2.7% of cancer-related deaths are caused by brain tumors. Primary brain tumors constitute about 2% of malignant diseases. Glioblastoma multiforme (GBM) is caused by the central nervous system-derived glial cells and is the most common (50–60%) form of primary brain tumors. In the elderly (45–70), GBM has higher possibility to emerge and has a high invasive phenotype showing a very rapid progression. The aim of this study was to investigate in vitro effects of selenium on human glioblastoma multiforme cells.

**Material and Methods:** In the present study, GMS-10 and DBTRG-05MG human glioblastoma multiforme cell lines were used as a model to examine, in the selenomethionine treated and non-treated groups; cell proliferation, cytotoxicity, and Ki-67 protein expression. Firstly, selenium entrance to the cells was assessed by measuring selenium in supernatants and lysates with graphite-furnace atomic absorption spectrometry (GFAAS). The effects of the organic source of selenium, selenomethionine, on cell proliferation and cytotoxicity, were assessed with WST-1 and lactate dehydrogenase (LDH) tests, respectively. Ki-67 protein expression was determined by western blotting.

The data were statistically analyzed using Man Whitney U test; p < 0.05 was considered to represent significant difference. The correlation between the SeMet levels which entered the cell and the parameters tested was analyzed using Pearson test.**

**Results:** This is the first study to examine SeMet effects on cell growth and death on GMS-10 and DBTRG-05MG cells. According to the results of our research, cells respond to seleno-L-methionine in a dose-dependent and time-dependent manner for both cell lines. The data obtained from the WST-1 test have shown that cell proliferation increased at 50 and 100 μM doses, which can be considered as low doses. With the analysis of the GFAAS results, SeMet levels of the cells were determined to be in accordance with viability and cytotoxicity tests.

**Conclusions:** In this study, SeMet, in the in vitro environmental conditions, has decreased proliferation and has shown cytotoxicity in a dose and incubation period dependent manner in both DBTRG-05MG and GMS-10 cells and these parameters are correlated in a large extent with the levels of SeMet entering the cells. In the view of the data obtained from this investigation, further studies focusing on the possibility of using SeMet against different types of GBM and in combination with prospect synergic compounds are considered to be worth-while.

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**SW04.S16–102**

**Synthesis and evaluation of antitumoral activity of some new pyrazolic compounds**

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The aim of our study was the synthesis and evaluation of new compounds with a potential selective antitumoral activity. The structures of the new synthesized pyrazolic derivatives were evaluated and their physical and chemical properties were determined by spectral analyses. The new compounds were tested for their antitumoral activity by several methods: apoptosis detection (Annexin V-FITC Apoptosis Detection Kit) and the cell cycle analysis by flow cytometry, clonogenic method, the detection and quantification of the proteins implicated in apoptosis (real-time PCR and Human Apoptosis Array kit, R&D System).

The most powerful antitumoral agent proved to be compound 4B, followed by 5A and 6A. 4B totally inhibited the tumoral clones’ growth at a concentration of 50 μg/ml. The inhibition process was less obvious in the case of 5A, 5B, 6A and 6B, while compounds 4A, 4C, 4D and 4E only slightly affected the colonies’ dimensions. The treatment of Hep 2 cells with 4B, 5B, 6B (50 μg/ml) induced higher expression of proteins involved in the extrinsic apoptosis pathway (FAS, FAD, TRAIL). Also, the pro-
caspase 3 levels were down-modulated, while the cleaved caspase 3 levels were up-modulated, maintaining the apoptotic pathways activated. The analysis of new compounds’ effect by flow cytometry showed that 4B, 5A, 5B and 6B (50 µg/ml) induced a drastic increase of G2/M phases in Hep 2 cells.

Our results showed that at least compound 4B might be considered for further investigation and eventually development of a new antitumor entity. Acknowledgement: TE-100/2010; PN-II-RU-TE 13/2011; POSDRU 89/1.5/s/60746.

SW04.S16–103

Study of protein content of mushrooms’ intracellular extracts having anti-inflammatory and anticancer activity

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In the last years many researchers have studied the possibility that extracts and isolated metabolites from mushrooms stimulate or suppress specific components of the immune system. Immuno-modulators can be effective agents for treating and preventing diseases and illnesses that stem from certain immunodeficiency and other depressed state of immunity [1].

We modulate growth conditions of mushroom culture, which lead to the sharp increasing of peroxidase activity of up to 300% and betta-glucosidase up to 200% at the same frequencies, as well as obtained increasing of protein content in extracts. Obtained differences have the differently directed character and depend from frequency and time of exposition of mm-waves [2,3].

We have determinate the anti-inflammatory activity of the above mentioned extracts of mushrooms on the widely used model of rat ear acute inflammation, induced by xylol. Intrapitoneal injection of an extracts from the irradiated by mm-waves cultures of the mushrooms are suppress an acute inflammation by 85%. Such effective influence of this extracts may be explain by sharp increasing of peroxidase activity in the cultures of a mushrooms and immune reply of rat organism on the mushroom extracts. Proteins of extracts was separated in PAAG and studied by HPLC analyze. Have been revealed increase of glutamine in 25 time in composition of some extracts, isolated from treated by mm-waves mushrooms cultures, which is probably responsible for such immune response of organism. In addition we investigate anticancer action of extracts on the HeLa cells in vitro. On the base of obtained results we suggest that immune reply of the body (rat or else) at the treatment of inflammation and cancer by the mushrooms extracts has a same mechanism. On the basis of obtained results we suggest to use treated by mm-waves mushrooms cultures as a pharmacological raw material, for resolving the problem of receiving effective drugs by easy and accessible methods. Our further investigation will show a correlation between mechanisms of anti-inflammatory and anticancer actions.

References


SW04.S16–104

Design of thrombospondin-binding peptides exhibiting strong anti-angiogenesis and anti-tumor properties

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Thrombospondin-1 (TSP1) is a ubiquitously expressed multimeric glycoprotein implicated in many pathophysiological processes making it a key actor in tumor environment and a potent target for the development of useful therapeutic tools against tumorigenesis and metastasis. As the anti-angiogenic properties of TSP1 are mediated by its C-ter domain interacting with the CD47 membrane receptor, we tried to identify peptides acting as specific antagonists for TSP1:CD47 interaction.

Peptides were designed using normal mode analysis (CHARMM), and docking experiments between TSP1, CD47 and CD47-derived peptides were conducted (GRAMM-X. Autodock). Molecular interactions were assessed by co-IP and ELISA binding assays. The consequences of peptide treatment regarding angiogenesis were investigated using in vitro (endothelial cell migration, invasion and tube formation) and ex vivo (mouse aortic ring assay) models. In vivo experiments were conducted using a homograft model of murine B16F1 melanoma, in which tumor morphology and vascularization were assessed through a multi-modal imaging approach using MRI and CI-scan correlated to histological analyses.

Molecular dynamics studies led to identify two CD47-derived peptides (TAX21 and its disulfide-bound analogue TAX2c) able to bind TSP1 specifically and to antagonize TSP1:CD47 interaction, as confirmed by co-IP and ELISA. Interestingly, these peptides inhibit angiogenesis both in vitro and ex vivo, while TSP1 (C-ter) interaction with CD47 is known to inhibit angiogenesis. Indeed, the proposed peptides induce in an original manner a molecular switch of TSP1 from CD47 to CD36 binding, responsible for a VEGF/VEGFR2-induced angiogenesis inhibition. Moreover, TAX2 peptides induce a strong tumor necrosis in vivo and substantially disturbed tumor vascularization. Finally, such CD47-derived peptides exhibiting strong anti-angiogenic activities in vivo, ex vivo and in vivo could therefore represent new exciting agents for cancer treatment.

A novel combined therapy to increase the sensitivity of breast cancer cells to tamoxifen

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The most important factor for the determination of endocrine responsiveness in breast cancer is the expression of ERα and ERβ hormone receptors in these cancer cells. In recent decades tamoxifen (TAM) therapy has been essential in the management
of patients with metastatic breast cancer. Its mechanism of action is based on its antiestrogenic effect, i.e. TAM blocks the action of the hormone that stimulates the growth of tumor cells. However, it is not useful for all types of breast cancers, but only in those whose cells have specific receptors for estrogen (ER+ cells). Breast tumors are heterogeneous at cellular level, and only 60% of breast tumors have this type of receptors. So, an interesting strategy for treating breast cancer would be found a combined therapy able to increase levels of the hormone receptors and therefore render breast cancer cells sensitive to therapy with TAM. Recently, we have observed that compounds that modulate the intracellular concentration of adenosine, such as dipyridamole (DIPY), greatly increase the antiproliferative effects of 3-O-(3,4,5-trimethoxybenzoyl)-(-)-catechin (TMCG), a synthetic antifolate derived from the structure of tea catechins. Due to the good results obtained with the combination TMCG/DIPY in several breast cancer cell models, we decided to test the effectiveness of this combination with TAM in ER-negative (ER-) breast cancer cells (MDA-MB-231). The results showed that the combination TMCG/DIPY/TAM caused a decrease in viable cell number greater than that observed with the individual TAM-treatment or the combination TMCG/DIPY. Then, we next studied whether the double combination TMCG/DIPY produced an increase in the expression of mRNA ERα in MDA-MB-231. The results showed, as expected, the absence of mRNA ERα in untreated cells; however in cells treated with the combination TMCG/DIPY we detected a significant increase in the expression of this receptor. This increase in mRNA was accompanied by a substantial increase in ERα protein. All together the data indicated that the combination TMCG/DIPY increased the sensibility of ER-breast cancer cells to antiestrogen therapy, which could be of importance in the management of patient with this type of breast cancer.

**SW04.S16–106**

IL-6 induced apoptosis and LDH release from K-562 cells

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IL-6 is cytokine with multiple roles in immunity, inflammation and cancer. Aim of this study is estimated in-vitro effect of IL-6 on expression several molecules on cell membrane of K-562 cells during induction of cell death. This research involved estimation of cytotoxicity by LDH release from cultures cells, analyses of changes in cell surface membrane molecule expression for CD45, CD30, CD38, and CD120 molecules by Flow cytometry in presence of diverse dose of IL-6 in time depending manner (2, 4, 6, 18, 24 hours). Apoptosis rate was also analyzed by Flow cytometry, using Annexin-V and PI staining. Using Western blotting assay we analyzed appearance of these molecule in cell culture supernatant. Results showed that IL-6 induce significant rate of apoptosis and necrosis in K-562 cell. IL-6 induced decrease of examined molecules on cell surface K-562 cells after treatment in comparison to pretreated values. However, decrease rate for examined molecules are not uniform and appear to be depending on their respective constitutive level on cell surface as well as molecule type. When we compared appearance of these molecules in supernatants we found that exist in high amount and correlates with decrease expression on cell surface. We conclude that these molecules can be shedding for cell membrane. Our process for investigated molecules proceeds apoptosis induction indicated that IL-6 as pro-inflammatory cytokine induced complex events on cell membrane during apoptosis induction.

**SW04.S16–107**

Redox-mediated P-gp transport activity in human CD19+ and CD19− lymphocytes

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It is known that redox-signaling mediates the multitude intracellular processes and can play an important role in regulation of multidrug resistance. In order to develop an adequate strategy of B- or T-type hemoblastosis therapy it is necessary understand the mechanisms of P-gp functioning in different populations of human lymphocytes.

In this work the influence of purine nucleoside analogs (fludarabine and cladribine), doxorubicin and vincristine on the reactive oxygen species (ROS) accumulation in the total human lymphocyte population as well as CD19+ (B-cells) and CD19− (T-and NK-cells) subpopulations was investigated and P-gp transport activity in these cells was estimated. So these enable to describe P-gp function under the redox-balance changing mediated by the anticancer drugs metabolism.

It was established that:

1) P-gp functionality in the process of drugs detoxification was exhibited in the maximum level on T-and NK-lymphocytes and in the minimum – on B-cells.
2) The viability of all investigated cell subpopulations after drugs exposure (during 15 hours) depends on the P-gp transport activity. Moreover, CD19+ cells are more sensitive to drugs compared with CD19− cells.
3) Regulation of the processes determining the cell viability (predominantly CD19+) after anticancer drugs exposure is redox-associated.
4) P-gp transport activity during 15 hours is regulated by redox-balance changing induced as the intracellular metabolism of the anticancer agents as the direct change of the peroxide level in the donor’s lymphocytes and significant inverse relationship was found for B-cells between ROS pool and P-gp functionality.

Thus, elevated ROS level in B-lymphocytes compared to T- and NK-cells determine the lower P-gp transport activity and as a result – higher sensitivity to the anticancer drugs action.

**SW04.S16–108**

Antineoplastic properties of pomolic acid isolated from Chamaenerion angustifolium

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Triterpene acids are one of the most promising groups of natural compounds for medicine. A number of drugs have been developed on their basis and used successfully in therapy. They have very broad range of biological activities including anti-inflammatory, analgetic, antipyretic, hepatoprotective, cardiotoxic and sedative activities. However, the list of useful properties is not limited by indicated activities and is broader.

Previously we have shown that in conditions of western Siberia rosebay willow-herb (Chamaenerion angustifolium) represents a natural source of triterpene acids [1]. Dynamics of target compounds accumulation during vegetative season and their distribution in different organs were investigated. It was found that at
the end of vegetation period the content of triterpene acids in leaves and stems of rosebay willow-herb is comparable to commercial sources of their production. Data were collected in the course of 3 years.

Besides ursolic and oleanolic acids which are wide spread in the plant raw material, we found pomolic acid for the first time in the rosebay willow-herb. Pomolic acid is considerably rare compound; the information about its biological activity is very poor. We were able to isolate pomolic acid in pure form (purity 95%) due to its high content in rosebay willow-herb plant raw material. Screening of mutagenic and genotoxic properties was performed via microbiological tests (SOS chromotest, Ames test). It was shown that neither pomolic acid nor its metabolites are mutagenic or genotoxic. Also screening of anticancer properties was carried out (MTT test). It was shown that pomolic acid has cytotoxicity against a number of cancerous cell lines and does not inhibit the growth of control cell line (human fibroblasts). Currently the mechanism of selective cytototoxicity of pomolic acid is studying.

Owing to above-listed effects, pomolic acid has excellent prospects for antineoplastic drugs, and rosebay willow-herb is very promising source of this target compound.

Reference


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**SW04.S16–109**

**Human proteome project for medicine**

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The Human Proteome Project (HPP) started in 2007 and officially launched in 2010 as the long-term collaboration of research groups in the field of cataloging all of the proteins coded by the human genome (Legrain et al., MCP, 2011). There was a shift from the analysis of information material (human genome) to the analysis and inventory of proteins - molecular machines operating in the human body.

Chromosome-centric HPP (C-HPP) was established as a wing of HPP implementing the chromosome-centric principle (Paiak et al., Nat Biotechnology, 2012, 221–3) prescribes the identification of the protein product encoded by each gene of the given chromosome. Russia has selected Chr 18, inspired by the intensive discussion occurring at the Moscow meeting in 2009, while considering the criteria for ranking human chromosomes according to their feasibility for the C-HPP: (a) minimum of protein-coding genes; (b) abundance of genes relevant to the diseases according to the available literary data; (c) lack of immunoglobulin-coding genes. Chr 18 contains totally 492 annotated genes and 276 protein-coding genes – so, about 276 master-proteins must be detected during the Russian part of pilot phase of C-HPP. Total number of protein species, coded by Chr 18, could be estimated as 20 000 included protein species, arising from nsSNP (SAP), alternative splicing and PTM.

Indicatively, the success of C-HPP depends on the new type of deliverables, which will be of use in the clinical diagnostics. To create the medical background for single chromosome, for example Chr 18, we have compiled information about 92 genes, reported in association with 105 diseases either in databases (GeneCards and OMIM (www.omim.org)) or in the relevant literature. According data about protein variances, we could expect the number of disease-related protein forms for Chr 18 is about 1 000. Within the framework of the C-HPP approach to the human proteome the top priority should be given to highly sensitive and specific (up to 10^{-18} M) detection of those proteins in injured tissues and plasma, which are absent in healthy human body (“0”) and typical exclusively for diseases (“1”). Alternatively, a “digital” signature would be consist of a protein present in a healthy human body and absent at some pathology. The digital response capturing can be featured by protein modification events, including SAP, AS, PTM and also fused onco-genes due to chromosome aberrations. That is fundamentally different approach: detection of protein modification events in frame of C-HPP instead master protein as biomarker in proteomics.

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**SW04.S16–110**

**Risk assessment of lung cancer development on the basis of mass spectrometry analysis of blood plasma metabolites**

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Lung cancer is one of the most common types of cancer in men, and is a leading cause of cancer-related deaths. Therefore, the identification of specific markers associated with risk of lung cancer development, particularly metabolites which are more easily assayed, would be extremely valuable. To this end, a comparative metabolomics study of blood plasma samples collected from patients with lung cancer (n = 100) and controls (n = 100) recruited in Moscow was performed.

All blood plasma samples were collected during multicenter case-control study carried out by Russian Oncological Scientific Center RAMS. After the removal of blood plasma proteins with methanol, the remaining plasma metabolite fractions were analyzed using direct mass spectrometry profiling method (micrOTOF Q, Bruker Daltonics Gmbh.). Numerous cancer-associated metabolites were detected and at least 70 metabolite ions with OR (Odds Ratio) values of 10–288 were found to be associated with the presence of cancer. While these metabolites were present at higher levels in cancer patients, particularly in the early stages of disease, they did not positively correlate with cancer progression. Based on these findings, this metabolomics study of blood plasma samples from cancer patients reveals numerous cancer-associated metabolites were present in the studied population, and these could be used as factors for calculating the risk of lung cancer development in addition to currently used risk factors.

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**SW04.S16–111**

**Pentamethinium salts as new type selective cytostatic agents for target of cancer signal pathways**

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Communication among cells is absolutely essential for multicellular organisms. All cell processes—including growth, death, and differentiation—depend on the action of signaling molecules and
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pathways. Changes in the system of cell–cell communication can often cause and/or enhance many pathological states such as cancer. These pathological signalling pathways are attractive targets for therapeutic intervention. An effective way of modulation, inhibition or activation, can be based on use of specific chemical ligands for individual components, e.g. signal molecules and their corresponding receptors. Their structure analysis indicated that some of these component (sulphate polysaccharide coreceptors), and sulphate steroid signal molecules can be recognised by same type of agent with sulphate selectivity such as pentamethinium salts.

In accordance with our expectations, analytical studies showed high affinity and selectivity of pentamethinium structure motif for sulphate polysaccharide coreceptors (chondroitine sulphate and heparan sulphate) and sulphate signal molecules (cholesterol sulphate, progolone sulphate and estrone sulphate) a coupled important spectral changes specific for the type of studied analytes. Next in vitro studies showed their high cytotoxicity for tested cancer cell lines against non-malignant fibroblasts cell lines. In addition, we observed significant correlation between sulphate selectivity of used pentamethinium salts and their cytotoxicity and cytotoxic selectivity for every tested cancer cell lines. Obtained results showed, that pentamethylum structure motif has high potential for development of new anti-tumor agents.

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SW04.S16–112
Increase in resistance of cancer cells in confluent cultures to survivin inhibitor YM155

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Survivin is one of the most important members of the family of inhibitors of apoptosis proteins (IAP), which regulates apoptosis and the process of mitosis. Survivin is selectively upregulated in human tumors, while it is absent in most normal adult tissues [1]. Its overexpression correlates with poor outcome and treatment resistance [2]. Thus survivin is a promising target for anticancer therapy [3]. Recently, YM155 is a small-molecule compound that inhibits survivin [4].

We investigated the cytotoxic activity of YM155 on tumor cells (human carcinoma cell line A431) and normal human fibroblasts. It was shown that YM155 caused the apoptosis of tumor cells in non-confluent cultures at nanomolar concentrations, however it did not damage the fibroblasts even at dose of 1 mM. According to these results YM155 can be considered as a promising anticancer agent. However, we found that A 431 cells being in confluent cultures after 96 hours of cultivation become completely resistant to YM155 even at high concentration (5 mM). We hypothesized that the confluent resistance is associated with reduced mitotic activity and that the resistance is reversible. It was revealed that cells located at the edge of a wound in confluent culture began to proliferate and was sensitive to YM155.

Thus confluent resistance of A431 carcinoma cells is reversible and associated with inhibition of cellular proliferation.

References


SW04.S16–113
Porous silicon nanoparticles for cellular delivery of anticancer molecules

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The use of porous silica nanoparticles (PSN) offers new perspectives in drug delivery and cancer therapy. PSN exhibit considerable potential for biological applications because of their biocompatibility, biodegradability and tunable porous nanostructure for drug delivery. Another advantage is the possibility to modify their external as well as internal surfaces. Nano-surface modification has proven to be beneficial for improving nanocarrier drug delivery and provides a wide range of functionalities. The aim of this study was to define the biocompatibility of the PSN and to settle a suitable experimental protocol to functionalize PSN (size up to 450 nm). Because PSN are particularly susceptible to air or water oxidation, to stabilize PSN we performed a partial chemical oxidation of the nanoparticles with Piranha Solution (H2SO4/H2O2 2:1) and tested their effect on human epidermoid cancer cells (H1355) and human peripheral blood lymphocytes (PBL). We then settled a PSN functionalization procedure. After oxidation, the PSN surface was treated with the chemical linker APTES [(3-aminopropyl)triethoxyxilane] in order to introduce a free functional ammino group. The functionalization of PSN before and after APTES treatment was examined using Fourier Transform Infrared Spectroscopy (FT-IR). The increase in intensity and slight change in the position of the peaks indicated that the PSN surface was functionalized and thus ready for the subsequent covalent binding of fluorochromes, peptides or drugs. The results obtained for biocompatibility of PSN-treated cells evaluated by MTT assay after 48 and 72 hours treatment showed substantially no cytotoxic effect on both PBL and H1355 cell, on the contrary a slight increase of vitality was observed already at 48 hours incubation. To evaluate instead the cellular uptake of the nanoparticles, APTES-functionalized PSN were reacted with TRITC (tetramethylrhodamine isothiocyanate) and after removal of TRITC excess incubated with H1355 for 24 hours. Analysis by confocal laser scanner microscopy revealed the presence of a large amount of PSN inside the cells and a smaller amount also into nucleous. These preliminary data highlighted that PSN are suitable vectors for cellular delivery and might provide further insights for clinical applications in cancer therapies.
Vibrational spectroscopy is a reliable analytical tool for the characterization of biological systems, as it provides accurate information at the molecular level. In particular, Raman confocal microspectroscopy of cell cultures allows a non-invasive probing of living cells [1] with a high sub-cellular spatial resolution, that enables observing and mapping the distinct cell components without the use of dyes or probes [2].

The aim of this preliminary work is to apply microRaman spectroscopy for probing living prostate cancer cells (PC-3), while preserving cell integrity and function.

MicroRaman spectra were recorded in a Renishaw InVia Raman microscope spectrometer, coupled to a RenCam CCD detector, using a Leica 100x lens. An exciting laser power of ca. 10 mW at the sample was applied, for 10 accumulations and 60 s of exposure time. Spectra of living PC-3 cells in exponential growth (3 x 10⁴ cells/cm²) were obtained in quartz windows, focusing on the cytoplasm, the membrane and the nuclei.

This study allowed to get access to the chemical composition of the tested in vitro cells. Further assays are ongoing, aiming at assessing the biodistribution and effect of Pt- and Pd-based anticancer agents, such as cisplatin [cis-Pt(Cl2(NH3)2)] and Pd2−Spm [Spm=H2N(CH2)3NH(CH2)4NH(CH2)3NH2], as a function of dosage and incubation time [3]. This will hopefully lead to a better understanding of the drug-cell interactions inducing cell-growth inhibition and/or death by apoptosis.

References

In vitro probing of human refractory prostate cancer cells by microRaman spectroscopy
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SW04.S16–115
CYP2D6 genotype and tamoxifen response in pre and postmenopausal Thai women with hormone responsive breast cancer
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Tamoxifen is effectively used as adjuvant therapy for hormone responsive breast cancer patients. Cytochrome P450-2D6 (CYP2D6) enzyme involves in metabolizing tamoxifen to active metabolites. CYP2D6 polymorphisms have been suggested to affect tamoxifen efficacy among Europeans and some Asians. In this study, we preliminary investigated the associations of CYP2D6 metabolizing enzymes genotypes with tamoxifen response measure as breast cancer-free interval time (refer to as recurrence). DNA extracted from patient blood was used for genotyping seven CYP2D6 single -nucleotide polymorphism using microarray-based pharmacogenetic testing. Genotype combinations were used to categorized CYP2D6 metabolism phenotypes as poor, intermediate, and extensive metabolizers (PM, IM, and EM, respectively, n = 48). Associations of CYP2D6 metabolism phenotypes with breast cancer-free interval were assessed using Kaplan-Meier analysis. Cox proportional hazards models were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). No association between CYP2D6 metabolism phenotypes and breast cancer-free interval was observed among all patients. However, the shorter disease free survival (DFS) was significantly shorter in post menopausal patients with PM phenotypes (homzygous variant TT when compared to those with heterozygous CT or homozygous CC) at nucleotide 100C>T and 1039C>T(CYP2D6*10)(Log−rank test p = 0.046). The association of increase the risk of recurrence, however was not significantly different among PM and IM
compared with EM phenotypes (HR3.48; 95% CI, 0.86–14.07; p = 0.080). The number of patients may be quite small and the study should be confirmed in a larger group.

SW04.S16–117
The role of polyamines in the design of anticancer drugs
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Biogenic polyamines (PA’s), putrescine, spermidine and spermine, are found in all eukaryotes and most prokaryotes. Owing to their important functions and recognized involvement in carcinogenic processes, they have been studied with a view to develop new anticancer therapeutic strategies. One of this approaches is their use as ligands for heavy metals ions such as Pd(II) and Pt(II), yielding stable polymeric chelates. Cisplatin, the parent compound of this type (Pt(C12(NH3)2) is one of the most widely used antineoplastic agents in clinical practice, but presents drawbacks, such as severe nephrotoxicity and acquired resistance. The coordination of biogenic amines to metal ions was found to lead to a significant increase of antitumour effects, probably due to a more efficient interaction with DNA.

The present study reports the use of these compounds in the design of effective anticancer agents toward human breast cancer cell lines.

Another approach is to test the effect of the naturally formed phenolic conjugates of polyamines, widely present in plants – phenolamides, hydroxycinnamic acid amides (HCAA) or phenylamides. These are either products of the polyamine catabolism or storage forms of polyamines or phenolic compounds. This type of phytochemicals is regularly consumed in the diet and have been characterised as bioactive agents displaying antioxidant, antibacterial, antifungal, antioxidative and radical scavenging activities.

This work describes the study of some pure phenolamides, from their characterisation by physical-chemical techniques (e.g. vibrational spectroscopy) to the determination of their biodistribution in human cells, aiming at an understanding of the tight structure-activity relationships (SAR’s) that underlie and control their biological role. Up to these days, most of these phytochemicals have been studied in the form of extracts and never in the isolated form, at the molecular level.

SW04.S16–118
Triterpene saponosides from Lysimachia ciliata – new perspective in cancer therapy
in vitro studies
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Despite of fact that process of carcinogenesis is well known and its mechanism is extensively studied there is still no effective cure for cancer disease. Traditional cancer chemotherapy frequently uses cytotoxic agents to destroy cancer cells at the expense of normal host tissue. Very important is search new chemotherapeutics agents that are effective will be incorporated with an outbreak of disease with minimal effect on healthy tissue. The quest for new drugs, new therapeutics strategies are needed. Triterpenoid saponosides are secondary metabolites derived from higher plants. Saponosides can be found in all parts of higher plants including roots, leaves, flowers and buds. Biological activities of saponosides are well known and they include: antimicrobial, anti-inflammatory, expectorant as well as anticancer action. Published data suggest the correlation between chemical structure of saponosides and their biological activities, however the exact influence on cancer cells is yet unknown. Subtle changes in substituents in the main chain of the chemical structure of saponins determine different biological activity.

Methods: In this study we analyzed saponosides with different chemical structure and compared their impact on the cancer and normal cells (Du-145 human prostate cancer cell line with medium metastasis potential, PC3 human prostate cancer cell line with high metastasis potential, PNT2 normal prostate cell line). Analysis of cells vital function include proliferation, morphology, invasiveness, mechanism of cell death, migration and cytoskeleton organization as well as cell elasticity.

Results: Preliminary result of our study indicate that these saponosides have a high selectivity in their effect on the examined cells and what is most the effect of studied saponosides is more pronounced on cancer cells than effect of mitoxantrone-commonly used in cancer therapy drug, in contrast to normal cells.

Conclusions: Triterpene saponosides are very interesting chemical compounds, their effect on cancer cells is promising, but requires further detailed research. Our data suggest that subtle changes in the chemical structure of saponins have a significant impact under biological role.

SW04.S16–119
Association of the MTHFR C677T polymorphism with toxicity in breast cancer adjuvant anthracycline-based treatment
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Introduction: Methyleneetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate metabolism pathway. MTHFR C677T polymorphism leads to amino acid substitution Ala222Val. As a result of this mutation, heterozygous and homozygous carriers of the 677T allele variant have a 30–40% and 60–70% reduced enzyme activity respectively. Recently, the MTHFR polymorphism was found to modulate the chemosensitivity of cancer cells to chemotherapy. Furthermore, the 677T variant is linked to severe toxicity during adjuvant treatment of breast cancer with cyclophosphamide, methotrexate, and 5-fluorouracil. The aim of our study was to reveal the association of the MTHFR C677T polymorphism with toxicity in breast cancer adjuvant anthracycline-based treatment.

Materials and methods: The case group comprised 54 patients with breast cancer. All patients received 4–6 cycles of anthracycline-based chemotherapy regimen with 5-fluorouracil, adriamycin, and cyclophosphamide (FAC). Treatment toxicity was assessed using NCI-CTC. Genomic DNA from peripheral blood was analyzed for identification of genotypes of the MTHFR using Allelic Discrimination Real Time PCR.

Results: During chemotherapy breast cancer patients developed some degree of gastrointestinal, hematological and cardiovascular toxicity. Cardiovascular toxicity was observed in 20% of patients. It was noticed that heterozygous and homozygous carriers of the 677T allele variant have a significantly higher risk for cardiovascular toxicity during chemotherapy administration compared to wild genotype carriers (OR = 4.48, 95% CI = 1.08–18.55, p = 0.03). Severe hematological and gastrointestinal toxicity developed in 13% and 39% of the cases, respectively.
dominant gastrointestinal toxicity types were nausea (95%), vomiting (31%), constipation (20%), diarrhea (3.8%) and stomatitis (3.8%); predominant hematological toxicity type – neutropenia. Severe hematological and gastrointestinal toxicity more frequently occurred in patients with CT or TT genotype compared to CC (p = 0.09).

Conclusions: The large individual variability for anticancer drugs in both disease outcome and chemotherapy toxicity risk makes the identification of pharmacogenetic markers that can be very promising to screen patients before therapy selection.

SW04.S16–120
PKC-alpha mediates the therapeutic effect of salinomycin on breast cancer cell lines
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Salinomycin is a monocarboxylic polymer antibiotic isolated from Streptomyces albous that has been recently identified as a selective inhibitor of breast cancer stem cells CSCs. The mechanisms by which this antibiotic act in human cancer cells to impair their viability, decrease proliferation and induce apoptosis are still unknown. For that reason, we have studied the effect of inhibiting PKC-alpha expression on the salinomycin treatment of two breast cancer cell lines (MDA-MB-231 and MCF-7). We found that salinomycin treatment decreased the migration capacity of both cell lines. This was more obvious when salinomycin was added to MCF-7 with down-regulated PKCalpha, in which case it completely abolished the migration capacity of the cell line, regardless of its concentration. These results suggest that salinomycin and PKCalpha might participate in the migration of MCF-7 cells through the same signaling pathways, since they show synergism in the inhibition of this process. We also studied the effect of salinomycin under PKCalpha inhibition on the invasion capacity of MDA-MB-231 cells and we found that combination of both significantly reduced the invasion capacity of the cells. We also found that salinomycin rapidly induces apoptosis in both cell lines. However, only in MDA-MB-231 there was a synergistic effect with PKCalpha inhibition, while in MCF-7 seems to be a PKCalpha-independent process.

Additional research is needed to determine exactly how salinomycin acts to kill cancer cells and to assess whether it can produce the same tumour-reducing power in humans as it does in mice and cell cultures.

SW04.S16–121
Mechanisms of anti-metastatic effects induced by dibenzoylmethane and its analogues on human breast carcinoma cells
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Dibenzoylmethane (DB), a minor constituent of the root extract of licorice, belong to the flavonoid family. Hydroxydibenzoylmethane (HDB) and hydroxymethyldibenzoylmethane (HMDB) are identical in structure to DB and possess a hydroxyl group and methyl group bonded to the aromatic rings, respectively. They inhibit cellular proliferation and induce apoptosis at a variety of cancer cells. Anti-metastatic effects of DB, HDB and HMDB on human breast carcinoma cells are generally unclear. This study aimed to clarify the molecular mechanisms by 12-O-tetradeca-
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ment of new 'smart drugs' for the treatment of glioblastoma multiforme.

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**SW04.S16–123**

**Chemical screening and cytotoxicity of some plants traditionally used as food in Armenia**

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Cancer is the second leading cause of death worldwide after cardiovascular diseases. Although great advancements have been done in the treatment and control of cancer progression, significant deficiencies for improvement remain. The anticancer properties of plants have been recognized for centuries. Natural therapy, such as the use of plant derived products, may reduce adverse side effects in cancer treatment. Attempts are made to characterize as chemopreventive agents the single constituents isolated from natural products. However, a few plant products are being used to treat cancer. The unique and very broad chemical diversity of medicinal plant extracts constitutes potential in the search for novel anticancer medicaments.

The aim of our study was to elucidate the anticancer properties of some plants traditionally used as food in Armenia. The ethanol extracts of *Rosa damascena* petals, *Rumex crispus* and *Vitis vinifera* leaves were characterized with their chemical constitution, cytotoxicity and antioxidant properties. The antioxidant activity measured by DPPH and ABTS methods decreased as follows: *Rosa damascena* petals > *Vitis vinifera* leaves > *Rumex crispus* petals. Using chemical analysis and TLC, we identified flavonoids, coumarins, tannins, floroglucides, phenolics, anthra-ene derivatives and anthocyanins in these plant extracts. The screening of 200 mkg of dried plant extract per ml of culture medium has shown their ability to suppress the growth of cultured malignant (Ehrlich Ascites Carcinoma, EAC) cells. The obtained data demonstrated that the extracts of *Rosa damascena* and *Vitis vinifera* had high cytotoxic effect (80–90% inhibition) against both the EAC cells and the primary culture of peripheral blood leukocytes of rats (nonmalignant cells).

The ethanol extract of *Rumex crispus* leaves inhibited the growth of EAC cells by 80% and of the non-cancer cells by 20%. Hence, our results manifest that it can be recommended as an agent for cancer treatment.

**SW04.S16–124**

**A new trifluorothymidine prodrug for treating cancer**

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A novel antitumor theranostic agent has been designed and synthesized. The agent represents a multifunctional construct containing a pyrimidine nucleoside antimetabolite (trifluorothymidine) and groups that are responsible for (i) increased affinity of the agent to tumor cells (residues of polyunsaturated fatty acid); (ii) efficient binding of the agent with the cellular membrane and penetration into a cell (polyethyleneimine modified with urocanic acid moiety); (iii) prolonged antitumor action of the agent (an acid-labile imidazole of 5-trifluoromethyl-2-deoxyuridine-5'-monophosphate); (iv) detection of the agent in the body using in vivo 31P NMR spectroscopy (amidophosphate group) and 19F magnetic resonance spectroscopy and tomography (CF3 group in the heterocyclic base). Using in vivo NMR spectroscopy it was shown that the theranostic agent can be detected directly in the organisms of animals models (in vivo 31P NMR: amidophosphate signal, δ ~ 12 ppm, 19F NMR: CF3 signal, δ ~ 101 ppm). Cytotoxic properties of the agent were studied by in vitro tests. The therapeutic construct was found to possess the highest cytotoxicity for the MCF-7 cell line (IC50 = 0.43 μM). The multifunctional construct containing 30 residues of urocanic acid and 40 residues of trifluorothydine 5'-monophosphate was tested against the murine Krebs-II ascites carcinoma, grown as an ascitic tumor. The intraperitoneal injection of the conjugates resulted in prolongation of the animals’ life and to the complete disappearance of the tumor after three injections.

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**SW04.S16–125**

**Programmed tumor cells death induced by recombinant analog of lactaptin**

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Previously we have found the proteins of human milk which are capable to induce the apoptotic death of human mammary adenocarcinoma cells MCF-7. Lactaptin – one of the pro-apoptotic proteins of human milk – was purified, characterized and identified as proteolytic fragment of kappa-casein [1]. Subsequently we constructed the recombinant analog of lactaptin (RL2) which induced apoptosis of MCF-7 cells [2]. Apoptosis of MCF-7 was characterized by activation of pro-caspase 7 leading to accumulation of the enzyme active form [3].

We have shown that apoptotic death of RL2 treated MCF-7 cells accompanied by phosphatidylserine exposure on the plasma membrane and activation of caspase 7. At that activation of DNAase DFF40 and oligonucleosomal fragmentation of DNA didn’t take place [2].

The analysis of biochemical markers of RL2 induced MCF-7 apoptosis was the main task of our investigation. To analyze the mitochondrial inner membrane permeability we have measured mitochondrial transmembrane potential (ΔΨ) of MCF-7. It was shown that RL2 induced dissipation of ΔΨ of MCF-7 cells.

To investigate the apoptotic caspases cascades (intrinsic or extrinsic) triggered by RL2 we performed analysis of initiator caspase 8 and caspase 9 activation. It was shown that RL2 induced the accumulation of both caspases 8 and 9 active forms. Thus we can suppose that the elements of intrinsic and extrinsic apoptotic pathway are involved in apoptotic death of the cells.

In this work we also have performed whole-genome HT assay for expression profiling of RL2 treated MCF-7 cells (Illumina). We have shown RL2 initiated the consecutive increase (8, 18, 28 hours) of mRNAs levels for the following gene groups: condensed chromosome kinetochore, microtubule cytoskeleton organization, regulation of mitotic cell cycle, cell cycle arrest. Moreover increasing level of p53 mRNAs as well as mRNAs of p53 upregulated MCF-7 genes were observed.
Identification of human MCF-7 cells proteins interacting with RL2 was accomplished by affinity purification coupled with tandem mass spectrometry method. We identified 3 proteins of cytoskeleton interacted with RL2: α and β chains of tubulin and α-actinin-1. Tubulin forms heterodimers and assembles into microtubules; α-actinin-1 is a homodimer that cross-links actin filaments into parallel bundles.

It is well known that cell cycle arrest at G2/M phase can occur when microtubules dynamic of mitotic spindle is suppressed. During G2/M arrest cells can die through caspase-dependent apoptosis or caspase-independent apoptosis upregulated by p53 [4]. Overexpression of p53 can lead to mitochondrial membrane permeabilization, loss of Δψ and activation of initiative caspase 9 [5]. Summarize, we have proposed the molecular mechanism of tumor cell death induced by recombaint analog of lactaptin.

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References

SW04.S16–126
BAl, a novel CDK inhibitor induces apoptosis or G2/M arrest according to p53 state of cancer cell
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A novel cyclin-dependent kinase (CDK) inhibitor, BAl has anti-cancer activity in various cancer cells. In this study, we evaluated the effect of BAl on apoptosis induction and cell cycle arrest in p53 wild or p53 mutant cancer cells. BAl induces caspase-dependent apoptosis in p53 wild cancer cells such as A549, Caki, HCT116 p53(+/-) and SK-Hep-1. BAl increased the expression of P53 and PUMA and nuclear accumulation of p53 in those cells. However, it arrested cell cycle to G2/M phase instead of apoptosis in p53 mutant such as H-1299, HCT116 p53(-/-), Hep3B and HT29. Transfection with P53 plasmid to p53 mutants and SK-Hep-1 increased BAl-mediated apoptosis instead of G2/M arrest. Long time treatment (72–96 hours) of BAl to p53 mutant induced p53-independent and caspase-dependent apoptosis. These findings collectively suggest that wether apoptosis was induced or cell cycle was arrested to G2/M phase by BAl treatment was potently dependent on p53 state of cancer cells.

SW04.S16–127
Effects of flavonoids on mushroom tyosinase and melanogenesis in mouse B16 melanoma cells
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Twenty seven flavonoids isolated from Dalbergia parviflora were screened for the inhibitory activity against mushroom and murine tyrosinases using L-DOPA as the substrate. Among the flavonoids tested, only four, namely Khrinone, Cajanin, 3'-hydroxy-8-methoxy vestitol and 3,8-dihydroxy-9-methoxy pterocarpan, showed potent inhibitory effect on mushroom tyrosinase while only 3,8-dihydroxy-9-methoxy pterocarpan effectively inhibits melanogenesis in B16-F10 melanoma cells at a concentration of 15 μM without being significantly toxic to the cells while reducing the melanin production by 35% and 60%, respectively. Moreover, the crude extract and some of the flavonoids showed an increase in melanin production in B16-F10 cells. These results suggested that the inhibition of mushroom tyrosinase activity does not correlate with that of mammalian tyrosinase or melanin production in cultured melanocytes.

SW04.S16–128
Modular nanotransporters efficiently deliver Auger electron emitters into nuclei of cancer cells
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Auger electron (AE) emitters possess extremely enhanced cytotoxicity when decaying in close proximity to nuclear DNA. Thus, when precisely delivered to the nuclei of cancer cells, they might be ideal for killing single tumor cells with minimal non-specific damage to normal tissues. Modular nanotransporters (MNTs), designed to transport drugs from the cell surface via receptor-mediated endocytosis and subsequent endosomal escape to the nucleus, are a potential platform for accomplishing this goal. We evaluated the potential utility of MNT for enhancing the nuclear delivery and cytotoxicity of two different AE emitters – the prototypical AE, 125I, and 67Ga, which possesses a nearly ideal half-life for ultimate clinical application for cancer therapy.

In this study we chose an MNT with epidermal growth factor (EGF) as the targeting ligand because EGF receptors are over-expressed on a great variety of cancer cells. For radioiodination we utilized the residualizing labeling reagent N-succinimidyl 4-guanidinomethyl-3'-(125I)iodobenzoate (125I)SGMIB), that yields labeled proteins that do not undergo appreciable deiodination in vivo. For labeling MNT with 67Ga, we selected 2,2',2''-(1,4,7-tri-azonane-1,4,7-triyl)triacetic acid (NOTA), which forms highly stable complexes with Ga(III).
MNT was labeled efficiently with both AE emitting isotopes. Both radiolabeled derivatives internalized effectively into EGFR-overexpressing cells, with more than half (55-60%) located within the cell nuclei after 1 h incubation. The cytotoxicity of both \([^{125}\text{I}]\text{SGMIB-MNT}\) and \([^{68}\text{Ga-NOTA-MNT}\) compared to labeled bovine serum albumin control was enhanced up to a factor of more than 3700 depending on the isotope, the cell line and its EGFR expression level. Moreover, both radiolabeled derivatives also showed 18-fold enhanced cytotoxic effectiveness over \([^{125}\text{I}]\text{SGMIB-EGF}\) or \([^{68}\text{Ga-NOTA-EGF}\).

Taken together, our results demonstrate that MNT is a promising platform for targeted radiotherapy utilizing AE emitting radionuclides, and we speculate that this approach will be most valuable in situations where the tumor specificity of the effect is most critical.

**SW04.S16–129**

**Targeted delivery of antitumor peptide lactaptin to tumors**

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The therapeutic efficacy of many anticancer drugs is limited by their poor penetration into tumors and by cytotoxic effects on healthy cells, which limits the dose of drug that can be administered to cancer patients. Docking-based targeting strategies use peptides, antibodies and other molecules that bind to tumor vessels and tumor cells to deliver more drugs to tumors than to normal tissues. Peptides are particularly well suited for drug targeting because they are small, easy to synthesize and typically non-immunogenic.

Lactaptin is the novel antitumor peptide that induces apoptosis of tumor cells in vitro, suppresses the growth of mice ascites and solid tumors and metastases in vivo, without obvious side effects [1].

In the present study peptides targeted to mice hepatocarcinoma A-1 (HA-1) were selected by phage display technology (Ph.D.®- Phage Display Peptide Library Kit, New England Biolabs). Two peptides – GLHTSATNLYLHGGS and SGVYKY–VAYDQWHGGS possessed the highest affinity for the tumor. Plasmid vectors to produce fusion proteins united addressed peptide and lactaptin were constructed. The ability of fusion proteins to suppress HA-1 tumor growth in mice with solid tumor transplants was studied.

**Reference**


**SW04.S16–130**

**Cytotoxic and antioxidant activity of 9-norbornyl-6-chloropurine – a novel carbocyclic nucleoside analogue**

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6-Chloropurines substituted at position 9 with variously modified bicyclic skeletons have recently been reported as anti-enterovirus agents [1]. Importantly, these compounds also showed considerable cytotoxicity and could be considered as candidates for the development of new anticancer drugs. \([{[\text{Ib}],\text{2,2,2]-Bicyclo[2.2.1.][hept-2-yl]-6-chloro-9H-purine}}\) (9-norbornyl-6-chloropurine, NCP) represents the most active compound from the series and thus served as a model compound for biochemical studies. In this work we explored cytotoxic profile and antioxidant activity of NCP aiming to understand its mechanism of action.

The selectivity of the cytotoxic effects of NCP was tested on human tumor and normal cells. Two leukemic cell lines (CCRF-CEM, HL-60), two solid tumor-derived cell lines (HeLa-S3, HepG2), and two normal cell lines (HUVEC-2, NHDF-Ad) were employed. NCP was found to be particularly cytotoxic to leukemia cells with a much lower effect on carcinoma cell lines and negligible toxicity to normal cells. The effects of NCP on the activity of some GSH-dependent enzymes suggest that NCP does not induce oxidative stress, on the contrary, it likely induces cellular antioxidant defense. In order to estimate the antioxidant potential of the compound in living systems, CCRF-CEM cells, two different methods for the antioxidant activity evaluation were used. First, intracellular reactive oxygen species were detected by means of a fluorescent probe 5,6-dichloromethyl-2,7-dichlorodihydrofluorescein-diacetate (CM-H2DCF-DA). Second, lipid peroxidation data were obtained by the HPLC-FLD detection of the thiobarbituric acid-malondialdehyde complex. Both methods yielded comparable results. NCP showed antioxidant activity and indicated its pharmacologic potential in the treatment of oxidative stress-related diseases.

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**Reference**


**SW04.S16–131**

**Nobiletin induces apoptosis and potentiates the effects of the anticancer drug 5-fluorouracil in p53-mutated SNU-16 human gastric cancer cells**

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Nobiletin is a typical polymethoxyl flavone from citrus fruits that has anticancer properties, but the molecular mechanism of its inhibitory effects on the growth of p53-mutated SNU-16 human gastric cancer cells has not been explored. In this study, nobiletin was found to be effective at inhibiting the proliferation of SNU-16 cells than other flavonoids. Nobiletin induced the death of SNU-16 cells through apoptosis, as evidenced by the increased cell population in the sub-G1 phase, the appearance of fragmented nuclei, an increase in the Bax/Bcl-2 ratio, the proteolytic activation of caspase-9, an increase in caspase-3 activity, and the degradation of poly(ADP-ribose) polymerase (PARP) protein. We found that the combination of nobiletin plus the anticancer
drug 5-fluorouracil (5-FU) reduced the viability of SNU-16 cells in a concentration-dependent manner and exhibited a synergistic anticancer effect (CI = 0.47) when 5-FU was used at relatively low concentrations. The expression of p53 protein increased after treatment with 5-FU, but not nobiletin, whereas the expression of p21WAF1/CIP1 protein increased after treatment with nobiletin, but not 5-FU. The cellular responses to nobiletin and 5-FU occurred through different pathways. The results of this study suggest the potential application of nobiletin to the enhancement of 5-FU efficiency in p53 mutant tumors.

**SW04.S16–132**

**Investigating expression levels of genes responsible for electron transport chain in uterine fibroid**

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Uterine leiomyomas, which severely threaten the health and quality of life of affected women are cause of pelvic pain, bleeding, infertility, sterility and the leading indication for hysterectomy. Familial and epidemiological studies provide significant evidence that genetic alterations play an important role in leiomyoma development. However, effective mechanisms of uterine leiomyoma has not fully understood. In this preliminary study, we aimed to investigate the expression profiles of genes response for electron transport chain in uterine leiomyomas and healthy uterine smooth muscle.

We studied uterine leiomyoma tissues and their paired adjacent healthy uterine smooth muscle tissues from six cases of uterine leiomyomas who were surgically treated in Ege University Hospital. Quantitative real-time PCR has used for investigation the expressions of 84 genes which response for electron transport chain in uterus leiomyoma.

PCR results showed that the expressions of ATP5G2 gene was significantly higher in uterine leiomyoma cases, as compared to the expression in uterine smooth muscles. Additionally, the expressions of ATP5J, PP1A, COX5A and NDUFS1 genes were significantly reduced in uterine leiomyoma cases, as compared to the expression in uterine smooth muscles.

These results show ATP5J, PP1A, ATP5G2, NDUFS1 and COX5A might plays an important role in the pathogenesis of uterine leiomyomas.

**SW04.S16–133**

**ABCG2, a polyspecific multidrug resistance protein: modulator screening studies**

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Breast Cancer Resistance Protein (or ABCG2) is one of the human ATP-Binding Cassette transporters. It is able to transport a wide spectrum of hydrophobic substrates using ATP hydrolysis as energy. It is involved in the multidrug resistance phenotype of cancer cells through the efflux of chemotherapeutic drugs which decreases their intracellular concentration and cytotoxic activity. Our group has concentrated efforts on the development of new ABCG2 modulators counteracting the resistance due to ABCG2 overexpression.

Inhibitor screening studies allowed us to demonstrate important contributions of methoxy substituents to both inhibitory potency and cytotoxicity. While the different classes of studied compounds (flavones, trans-stilbenes, chalcones and chromones) indicated that methoxy groups on both sides of the molecule strengthened ABCG2 inhibition, some substituted chalcones showed that some methoxy groups were also critically involved in cytotoxicity [1]. This was the first time that structure-activity relationships were reported about the cytotoxicity of ABCG2 inhibitors; we may assume that such information is quite important for designing new pharmacophores aimed at running future in vivo studies. These studies highlighted a lead compound: chromone 6 g with a high affinity and low cytotoxicity, leading to a very high therapeutic ratio, around 1000 [2]. Chromone 6 g therefore, is now investigated in preclinical studies in SCID mice bearing human xenografts.

On the other hand, since inhibitor had given poor results in clinical studies we recently started to search for a new strategy so-called ‘Achille’s heel’, which consists in selectively killing cancer cells overexpressing ABC transporter. This strategy had been demonstrated for P-gP and MRP1 and we recently started to focus on similar ABCG2 modulators.

Overall, these modulator studies helped us to better understand ABCG2 mechanism and allowed the development of different strategies to counteract ABCG2-related resistance.

**References**


**SW04.S16–134**

**Trigona sirindhornae propolis reduces progression of head and neck cancer cell lines**

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Propolis is a derivative product from plant resins collected by honeybees. It has been proved for multi-biological functions including anti-tumor development and progression. Head and neck squamous cell carcinoma (HNSCC) is one of cancer that shows high incident rate worldwide and in Thailand. There is no any effective treatment strategy for the patients, result to the mortality rate is not decrease until now.

This study aims to explore anti-proliferative and migration of propolis extracts from *Trigona sirindhornae* (high abundant bee in Thailand) on primary and metastatic HNSCC cell lines. The honeybee was collected and sequentially extracted by ethanolic method. The propolis extracts were indentified its contents using high-performance liquid chromatography (HPLC). The purified propolis was used to treat with HNSCC cell lines. Cell proliferation and migration were determined using MTT and wound healing assay, respectively. Propolis extracts showed anti-proliferative effect on HNSCC cell lines as dose dependent manner and 200 μg/ml propolis could significantly reduce cell proliferation of those cell lines compared to individual controls. Moreover, we found that propolis extract decreased cell migration of metastatic HNSCC cell line when non-cytotoxic dose was used. *T. sirindhornae*...
 ROS-induced apoptosis of human melanoma cells using biocompatible polyelectrolyte nanocapsules as a carrier of cyanine-type photosensitizer

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Melanoma is the most serious skin cancer, accounting for about 80% of deaths and patients develop metastases with the 5-year survival rate being only 14%. Currently, healing options such as chemotherapy, radiotherapy and immunotherapy are increasing but the therapeutic outcome is still limited because of the resistance of melanoma cells to these agents [1]. Therefore, there is still ample room for alternative treatment strategy, i.e., the generation of reactive oxygen species (ROS) followed by apoptosis induction after photodynamic therapy (PDT) of cancer cells [2]. The third generation of photosensitizers, e.g., indocyanines encapsulated in polymeric nanocarriers, is of particular importance in design of anticancer therapeutics with enhanced both tumor selectivity, and biocompatibility [3–4].

In our studies we fabricated novel IR-780 loaded multilayer nanocapsules by means of sequential adsorption of opposite charge polyelectrolyte (PE) layers on the nanoemulsion liquid core (layer-by-layer (LbL) approach) according to [5]. For this purpose we applied an anionic docusate sodium salt (AOT used as emulsifier) and a cationic poly-L-lysine hydrobromide (PLL applied as polycation) surface complex (AOT/PLL). Chloroform with dissolved cyanine was used as the oil phase. PEG-ylated external layers were prepared using poly-L-glutamic acid sodium salt and poly(ethylene glycol) (PGA-g-PEG). All nanocarriers, with the PE shell and average size <100 nm (as analyzed by DLS, AFM and SEM), were subjected to in vitro biological analysis on human malignant melanoma Me45 cell line, i.e., cyto- and photocytotoxic activity evaluated by MTT-based cytotoxicity assay as well as the ROS generation accompanied by a significant upregulation of Hsp70 expression and protein degradation (oxidative stress markers’ studies). Intracellular distribution of free and encapsulated indocyanine IR-780 was performed by confocal laser scanning microscopy (CLSM). The apoptotic cell death was evaluated by TUNEL method and trypan blue staining. Immortal human skin keratinocytes (HaCaT) were used as reference normal cell line.

The studied nanocarriers demonstrated good colloidal stability, profound IR-780 encapsulation capacity and enhanced uptake of IR-780 in comparison to the free photosensitizer. Further biological studies provided on the Me45 and HaCaT cell lines proved that obtained PEG-ylated polyelectrolyte nanocapsules are nontoxic in the dark. Encapsulated IR-780 showed improved ability to produce ROS and its excitation caused a higher decrease of the Me45 cells viability – generated ROS oxidized cellular proteins and involved changes in Hsp70 expression – than its native form. The cellular death analysis revealed apoptosis in melanoma cells after photodynamic reaction treatment with encapsulated cyanine. Therefore, the obtained IR-780 loaded nanocapsules present considerable potential for application as useful and effective photosensitizer delivery systems in PDT of melanoma and skin cancers.

References

SW04.S16–135

Effects of quercetin on experimental cancer in rats following oxidant/antioxidant balance

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Various types of antioxidant compounds are active against reactive oxygen species produced during metabolism and reduce oxidative damage of DNA, proteins, amino acids, and lipids. Quercetine is a type of polyphenol and a renowned antioxidant that effectively prevents cancer by removing free radicals.

In this paper we try to demonstrate in vivo the biological action of quercetine and highlighting the role of the concentration and the time of the administration of this compound to keep the antioxidant/pro-oxidant balance latter being responsible for the toxicity of the investigated flavonoid.

For this purpose were used as experimental animals (Wistar rats) bearing tumors (Walker carcinoma and RS-hepatoma) treated three times a week with 50 mg/kg body quercetine in DMSO at 7 and 21 days respectively from the tumoral graft, for 3 weeks. We investigated biochemical parameters of serum oxidative stress (lipid peroxides, copper-oxidative activity of ceruloplasmin, albumin thiol groups and the ability of serum to reduce iron) in dynamics of treatment. And in tissue sample with flow cytometry analysis we follow the S-phase fraction, proliferative index, and DNA index. All treated groups revealed some therapeutic benefits in contrast with control, consisting in loss of aneuploid population, decrease of DNA synthesis to the normal rate and accumulation of cells into G1 phase.

The obtained results indicate an antioxidant action of compound in the first week of treatment followed by a number of side effects that led to the death of the animal along with the extension of the treatment. The antioxidant capability of quercetine strongly depends on the intracellular availability of GSH. Thus, the capability of quercetine to induce apoptosis in cancer cells undoubtedly renders this molecule an interesting tool in the oncology field. Any compound that attends oxide-reduction metabolic reactions may come from an antioxidant to a pro-aggressive oxidizer when is outdated and time scales in the optimum necessary treatment.
SW04 Molecular Mechanisms of Disease

SW04.S16–137
Troger’s bases bearing two hydrazone units as new cytostatic agents
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Treatment of cancer is currently complicated and demanding with a number of unpleasant side effects. The increasing resistance of cancer towards drugs is also problematic; for these reasons searching for new anticancer compound is still required. One of the most promising types of cytostatic agents is based on hydrazones. The mechanism of their anticancer action has been assumed to be based on the chelation of metal ions, inhibition of the enzymes responsible for the biosynthesis of RNA and DNA as well as other biological processes, intercalation to DNA, disruption of the cellular metabolic processes or formation of the redox systems producing reactive oxygen species. It is expected a combination of the above mentioned mechanisms.

We designed and synthesized Tröger’s base derivatives bearing two hydrazone units that were separated by a rigid skeleton with a V-shape geometry. Anticancer activity of prepared derivatives were tested on human promyelocytic leukemia cells (HL60) and mammary carcinoma cells (4T1). The most active compounds have IC50 values lower than 1 μM. Complexation studies demonstrated that these compounds are able to bind Cu, Ni and Fe.

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SW04.S16–138
Predictive significance of thymidylate synthase expression in non-small cell lung cancer (NSCLC)
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Chemotherapy has been an important modality of treatment of NSCLC. The assessment of predictive molecular markers could help to estimate the response rate to chemotherapy. We assessed a panel of potential predictive markers of adjuvant chemotherapy in patients who underwent surgery for NSCLC. Here we publish the results for thymidylate synthase (TS).

TS catalyzes conversion of deoxyxuridylate (dUMP) to deoxy-thymidylate (dTMP), which maintains the dTMP pool critical for DNA replication and repair. Therefore TS has been of interest as a target for cancer chemotherapeutic agents, such as 5-fluorouracil (5-FU) and some folate analogs.

The studied group consisted of 70 patients with NSCLC (median age 61.5 years at the time of surgery, 51 men, 19 women), who had undergone curative lung resection. Paired lung tissue samples taken directly from the tumor and from adjacent, histologically cancer-free lung tissue (control) were immediately frozen to −70°C and stored until usage. All the samples were histologically verified as NSCLC [squamous cell carcinoma (SCC) 39, adenocarcinoma (AD) 27, other subtypes 4].

For quantitative estimation of TS gene expression was used RT real-time PCR method with UPL probes. Transcript levels were normalized against GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

We did not record differences in TS expression between tumor and normal lung tissue. On the contrary, histological subtypes AD and SCC differ significantly with higher TS transcript level in AD (p = 0.032). Furthermore, we found no relationship between TS expression and cytotoxicity reaction. The data were also evaluated in the relation to disease-free interval (DFI) and overall survival (OS) in NSCLC and its histological subtypes as well as in stage groups. Despite any of the results did not reach statistical significance, it is noteworthy that NSCLC patients with stage 2 and lower TS mRNA level have longer OS (p = 0.078).

To sum up, our results indicate that TS expression level lacks sufficient predictive value in our group of NSCLC patients.

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SW04.S16–139
Prospects of anticancer therapy by lactaptin
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Lactaptin, a human milk-derived protein, induces apoptosis in cultured tumor cells. Recombinant analog of lactaptin RL2 was designed. The sensitivity of mouse and human cancer cells was tested to determine the most reliable models. HA-1 mouse hepatocarcinoma and MDA-MB 231 human breast adenocarcinoma, which had the highest sensitivity to RL2, were selected to investigate RL2 antitumor activity in vivo.

The significant delay of tumors growth in mice A/Sn with inoculated HA-1 and SCID mice with inoculated MDA-MB 231 was showed at RL2 treatment. Histomorphometric analysis indicated a threefold reduction of spontaneous metastases in the liver of RL2-treated mice with HA-1 solid tumor transplants in comparison with control animals. Repeated RL2 treatment substantially prolonged the lifespan of mice with intravenously injected tumor cells.

Mechanism of apoptosis induction by RL2 was studied. It was shown that RL2 effectively penetrated into cells, binded α and β chains of tubulin and β2-actinin-1 and induced dissipation of mitochondrial membrane potential. Activation of initiator caspases 8, 9 and effector caspase 7 was also demonstrated after processing human breast cancer MCF-7 cells by RL2.

The possible model of cancer cells apoptosis induction by recombinant lactaptin analog LR2 was proposed.

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Abstracts

**SW04.S16–140**

**Selection of DNA aptamers regulating STAT5B, a protein involved in leukemias**

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Leukemias are due to abnormal cell proliferation, which is the result of intracellular over-expression or excessive activation of protein due to an oncogenic event. Still today, it is necessary to find new therapeutic molecules, which specifically target these proteins.

STAT5, via the JAK/STAT signalling pathway, controls fundamental cellular processes, including cell survival, proliferation and differentiation [1,2]. To struggle against tumorigenesis, JAK/STAT signalling pathway has to be inhibited. The aim of this project is to target specifically STAT5 factors to restore healthy signal transduction.

We generate aptamers by an iterative in vitro selection. Aptamers are short-structured single strand DNAs or RNAs that bind with high affinity and specificity to their target [3]. Once STAT5B recombinant proteins are produced, they are subjected to SELEX process. The number of rounds depends on various parameters [4]. After seven rounds, two sequences are retrieved.

The specificity and affinity of these aptamers are assessed by fluorescent immunoassays. Binding affinity and kinetics of interaction are characterised by SPR. Aptamer antiproliferative effects are determined by evaluation of the growth of cells depending on STAT5.

Finally, we develop several assays aiming at understanding the mechanism of aptamer action on STAT5B such as: Phosphorylation measurement, EMSA.

Aptamers are now emerging therapeutic tools; they exhibit significant advantages relative to protein therapeutics [5].

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**SW04.S16–141**

**Design and optimization of targeted therapeutics with precisely controlled pharmacokinetic and biodistribution properties**

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It is widely believed that nanotechnology will have a revolutionary impact on the treatment of cancer. A particularly promising application of nanotechnology in cancer therapy is the use of targeted nanoparticles (TNPs). TNP approach allows to enhance the accumulation and uptake of anticancer agents at specific sites, thus limiting exposure to off-target tissues and improving the therapeutic index of conventional chemotherapeutic drugs and molecularly targeted therapeutics. A major obstacle preventing translation from basic research is the inability to achieve the optimal interplay of physicochemical parameters that confer tumor targeting, evasion of particle clearance mechanisms, and controlled drug release.

With the aim to solve this problem we created a Medicinal Nanoengineering® platform that enables predictable, rapid design and optimization of targeted therapeutics. This platform was developed in Massachusetts Institute of Technology and BIND Therapeutics, Inc [1], and then was transferred to BIND(RUS), LLC with the view of creating new generation of drugs in Russia. Our technology is focused on the design of combinatorial libraries of TNPs with precise and systematically varied biophysical properties such as particle size, surface properties, ligand density, active pharmaceutical ingredient (API) load and release profile, using a unique self-assembly nanoparticle fabrication process to optimize each product-for example, to balance circulation time with effective targeting and binding for a particular cell or tissue target.

The developed approach of screening and optimizing physicochemically diverse TNPs is potentially applicable to a broad range of API classes for which preferential tumor accumulation can increase efficacy and decrease toxicity.

**Reference**


**SW04.S16–142**

**Cages for cancer cells, a new approach to block cell division**

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During mitosis the nuclear lamina, polymeric structure located underneath the inner nuclear membrane, have to be disassembled. The disassembling process is mediated by phosphorylation of the major nuclear lamina component, laminA at serine22 by mitotic kinase CDK1. Phosphorylation leads to depolymerization of laminA fibrils and decomposition of the nuclear lamina.

Our results demonstrate that prevention of lamina disassembly blocks mitosis in all tested cancer cell lines and leads to senescence. To stabilize nuclear lamina we have to accumulate laminA in the lamina by using Zmpste24 inhibitor lopinavir and to mitigate laminA serine22 phosphorylation by flavopiridol, CDK1 inhibitor. Permanent nuclear lamina works like a cage preventing nuclear division in cells.

However it is reasonable to suggest, that cages for cancer cells can be constructed at different levels. For example, using cancer cell specific nanoparticles we could create the cage around the whole cell eliminating the space for division. Such nontoxic cage will work despite the mutations present in cancer cells at any stage of cancer progression.
SW04.S16–143
Oridonin inhibits RNA transportation to reduce glioma cell growth via down-regulation of RanGAP expression
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Malignant gliomas are characterized with an intrinsic resistance to conventional therapies. Recent studies have demonstrated the median overall survival rate for glioblastoma patients to be 10.2–14.6 months. However, the prognosis remains poor, even with treatment that combines surgery, radiation, and/or chemotherapy. Therefore, it is important to develop a new strategy for treatment of glioblastoma. In tumor cells, RNA production is maintained at an abnormally high level to facilitate the cell growth. The most likely hypothesis in this study assumes that control of RNA export from nucleus to cytoplasm may interfere with the growth rate, and even lead to cell death in tumor cells. RanGTP bears RNA to form a complex in nucleus, then RanGTP would be hydrolyzed to RanGDP by RanGAP, and result in the release of RNA into cytoplasm. Both of free form and SUMOylated form of RaGAP are able to hydrolyze RanGTP. In this study, 5–30 μM of oridonin, a natural diterpenoid compound isolated from traditional chinese medicine R. rubescens, induced U87MG cells apoptosis and RNA accumulation in nucleus via a dose-dependent manner at 12-hr time point, suggesting that oridonin-induced cell death may result from impairment of RNA transportation. The immunofluorescence stain showed that treatment of 20 μM oridonin for 6 hours induced RanGTP accumulation in nucleus and down-regulated the amount of nuclear membrane-bound RanGAP. Furthermore, the immunoprecipitation data indicated that the free form and SUMOylated form of RanGAP were both decreased after treatment of 20 μM oridonin for 6 hours. These results strongly suggest that oridonin may induce the reduction and redistribution of RanGAP protein to suppress RanGTP hydrolyzation, then result in RNA accumulation and cell death in U87MG glioma cells. Based on these results, we demonstrated for the first time that down-regulation of RanGAP protein level by oridonin results in RNA accumulation in nucleus which leads U87MG cells to undergo apoptosis. The anti-tumor effect by impairment of RNA transportation exhibited by oridonin warrants further investigation toward clinical application in glioma therapy.

SW04.S16–144
Targeted therapy of human Glioblastoma using saporin delivery approach
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Glioblastoma represents one of the most worst adult tumor forms, scarcely responding to chemotherapy and with rapidly onset of recurrences. This tumor is particularly expressing membrane nucleolin and the splicing variant of EGFRvIII, very interesting and peculiar markers, that can be the target for specific delivery of new anticancer candidate molecules. We have constructed an expression plasmid containing the leading sequence of ASI1411, an aptamer DNA already demonstrated to bind membrane nucleolin that is currently under clinical trial, carrying the gene of the ribosome inactivating protein saporin. This aptamer-based gene delivery system has been tested on the glioblastoma cell line U87. The results obtained demonstrate that the cytotoxic activity of this molecule is dependent on saporin toxicity and occurs in a concentration range at least three orders of magnitude lower than that of the the free aptamer, with an IC50 around 1 nM. The toxic effect on cells involves caspase activation, although nuclear fragmentation revealed by nucleosome release and cytokeratin does not correlates with this process, suggesting other forms of cell death such as autophagy. We are also producing a recombinant scFv-anti EGFRvIII-saporin immunotoxin in the yeast Pichia pastoris. The production of recombinant saporin-based immunotoxins presents difficulties due to the intrinsic toxicity of saporin on the host cells, thus needing solutions for the improvement of productions. Fermentation approaches for the reduction of toxicity and degradation problems will be discussed. Perspectives: application of a dual targeting system involving both nucleolin and EGFR targeting will be in our opinion appealing for the therapy of this particularly aggressive cancer.

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SW04.S16–145
Potent angiogenic activity of prohaptoglobin and detection of prohaptoglobin in human cancer serum
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Haptoglobin (Hp), a major acute phase plasma protein, is synthesized as a precursor (proHp) which is a single polypeptide containing α- and β-subunit region, thereafter it is proteolytically cleaved to give separated α- and β-chains. Mature Hp, a heterotramer or polymer of (α-β)n, is yielded by linking the two chains through disulphide bonds. Although most Hp is secreted into the circulation as a mature form, a part can be released as a proHp. However, the biological function of proHp remains unclear. In the present study, we investigated the effect of proHp on angiogenesis in vitro and ex vivo. The gene expressions of VEGF and VEGFR2 as well as tubular structure formation on Matrigel and cell migration were enhanced in the HUVECs treated with conditioned medium from proHp-overexpressing COS7 cells, while the culture medium of control COS7 cells did not affect the angiogenic enhancement. When proHp was knocked down by specific siRNA transfection, the angiogenic activity of the conditioned medium was attenuated. In addition, ex vivo mouse aortic ring angiogenesis assay showed the proHp-increased endothelial sprouting and branching. The angiogenic activity of proHp was shown at low concentration of 0.1 μg/ml, while mature Hp purified from normal human serum did not show the activity even at 10-fold higher dose. Interestingly, proHp was detected in the serum from cancer patients, while little detected in normal human serum. Furthermore, proHp bound to haemoglobin very weakly, in contrast to strong binding of mature Hp. These results suggest that proHp is different from mature Hp in the structure and function, and its specific function might be applied as a novel biomarker for diseases associated angiogenesis.
SW04.S16–146
Effects caused by cyclophosphamid on microelement level and antioxidant system activity in rat tissues: a possibility for supporting therapy of cancer patients by microelement preparations
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The work is devoted to the study of an action of mutagen and carcinogen cyclophosphamide (CP) used for cancer treatment (40 mg/kg, single i.p. administration) on macro- and microelements level (30 elements), along with lipid peroxidation (LPO) indicators’ level and activity of antioxidant enzymes (24 hours after administration). It is found that CP i.p. administration doesn’t influence macroelements’ level in organs (brain, kidneys, liver), but drops dramatically a level of seven essential microelements: iron, zinc, manganese, molybdenum, cuprum, cobalt, and selenium. Under these conditions, CP doesn’t change chromium and iodine level, and boron, silicon, nickel, lithium, and arsenic level as well (although vanadium level is decreased statically in liver). It is shown that CP i.p. administration doesn’t influence a content of toxic microelements in rat organs. At the same time, CP i.p. administration influences weakly organism antioxidant defense, namely, activity of red blood cell peroxidase, total blood serum antioxidant activity, blood serum ceruloplasmin level, blood serum lipid hydro peroxides’ level, and red blood cell catalase activity. In rat organs (heart, liver, kidneys, adrenal glands, spleen), a level of LPO indicators – dien conjugates (DC) and malonic dialdehyde (MDA) are changed weakly, although thymus and brain DC and MDA levels appeared to be increased. Thus, CP administration resulting in genome instability and liposome lability demonstrates weak prooxidant properties and drops a level of seven essential microelements. It seems therefore that cancer patients should get supporting therapy using these seven essential microelements’ preparations just after chemotherapy to keep their antioxidant defense active and to recover.

SW04.S16–147
Development and in vitro efficacy of liposomes carrying anthracyclines and emetine for enhanced therapeutic effect
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Background: The anthracycline daunorubicin is the drug of choice for treatment of several different leukaeasms. We have previously shown an enhanced therapeutic effect of daunorubicin against acute myeloid leukaemia (AML) when combined with a protein synthesis inhibitor. This was seen both on AML cells in vitro and in AML-bearing mice. This effect was particularly interesting, since findings suggest that protein synthesis inhibitors could reduce toxic side effects of anthracyclines. To further reduce side effects, and to ensure delivery of both drugs to the target, we wanted to encapsulate them in one nano-carrier with functionalized surface to ensure targeted delivery to tumour-burdened tissues or organs.

Methods: Liposomes consisting of hydrogenated phosphocho- line, cholesterol, PEGylated phosphocoline and folate-PEG- phosphothanolamine were loaded with anthracyclines and/or emetine by the acid precipitation method. The drug load into the liposomes was quantified by HPLC, and the different liposomal formulations were tested for ability to induce cell death in FR 2-presenting AML cell lines. Flow cytometry and confocal microscopy was used to investigate the liposomal/drug uptake in cells.

Results: We managed to incorporate both anthracyclines and emetine in liposomes by the acid precipitation method. The incorporation did not alter the size of the liposomes, but increased their zeta potential, suggesting that some drug was associated on the outside of the liposomes. The combination of anthracyclines and emetine in the liposomes enhanced AML cell death. Additional drug loading and efficacy was found when FR2-expression was enhanced in AML cells by pre-treatment with methotrexate.

Conclusions: We here present a drug formulation that can prove to be more effective towards cancers with mutations associated with poor outcome like non-functional p53. Drug loading was efficient, and could be done in one step. The targeted uptake of liposomes in cancer cells suggest that these can reduce the severe side effects of anthracyclines seen in for instance the intestine of AML patients undergoing intensive chemotherapy. The next step will be to prove their efficacy in animal models for metastasising prostate cancer and AML.

SW04.S16–148
Effect of Amaryllidaceae alkaloids haemanthamine and haemanthidine on cell viability, apoptosis and cell cycle progression in human T-lymphoblast cell line
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Aim: To evaluate the effects of haemanthamine and haemanthidine, an isoquinoline alkaloids extracted from an Amaryllidaceae family plants, on human leukemic cells viability, cell cycle distributions and apoptosis. We also aim to clarify cellular and molecular mechanisms involved in the haemanthamine and haemanthidine-induced cell death in p53-null Jurkat cells.

Methods: In this work, we measured the cytotoxic effects of micromolar concentrations of Amaryllidaceae alkaloids haemanthamine and haemanthidine on human leukemic Jurkat cells. Cell viability was determined using a dye-free system Casy Cell Counter. For apoptosis detection we used flow cytometry-based quantification of Annexin V-FITC and propidium iodide dual staining. Results were cross-verified using the fluorescence microscopy. Activity of caspases 3 was determined by Caspase-Glo3/7 luminescent assay. Dissipation of the mitochondrial membrane potential after treatments were monitored using JC-1. The cell cycle was analyzed by flow cytometry.

Results: Our data showed a significant decrease in the percentage of viable cells after 24h treatment with both haemanthamine and haemanthidine. Furthermore, an increase in apoptosis was observed when cells were treated with both compounds. However, in haemanthidine-treated cells, there was a marked increase in Annexin V positive cells (cells that are in early apoptosis) contrary to haemanthamine-treated. Cells treated with both haem- anthamine and haemanthidine for 24 hours had a reduction of the mitochondrial membrane potential. There was sharper decrease in the percentage of cells displaying an intact mitochondrial...
L-DOPA decarboxylase (DDC) upregulation correlates with aggressive breast and prostate tumors, representing a novel biomarker for the accurate prognosis of breast and prostate cancer patients’ outcome

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Introduction: Breast (BC) and prostate cancer (CaP), constitute the most common types of malignancy in women and men, respectively. L-DOPA decarboxylase (DDC) catalyzes the decarboxylation of L-DOPA to dopamine. The clinical value of DDC has already been highlighted in neuroblastoma, small cell lung cancer and malignancies of gastrointestinal tract. In this study, we analyzed DDC expression levels in BC and CaP, in order to evaluate its ability as a biomarker for patients’ prognosis and management.

Methods: Total RNA was extracted from 143 BC specimens and 89 non-malignant compartments, as well as from 70 CaP and 57 benign prostatic hyperplasia (BPH) specimens. cDNA was produced by reverse transcription and a SYBR Green based Real-Time PCR assay was performed for the relative quantification of DDC expression levels using the comparative Ct (2^-ΔΔCT) method.

Results: DDC mRNA levels show a significantly negative correlation with ER (p < 0.001) and PgR (p < 0.001) levels and a positive correlation with Ki67 index (p = 0.034). Furthermore, the unfavorable nature of DDC expression in BC was confirmed by the statistically significant DDC upregulation in high-grade tumors (p = 0.029). Focusing on CaP, a significantly higher DDC expression in CaP patients compared to BPH ones (p < 0.001), was observed. Moreover, unregulated DDC expression was identified in late-stage (p = 0.032) and dedifferentiated tumors (p = 0.022) as well as in high serum PSA patients (p = 0.025). Finally, Kaplan-Meier analysis highlighted the significantly shorter disease-free survival of the CaP patients expressing high DDC levels (p = 0.015).

Conclusion: Our results reveal the ability of DDC to serve as an unfavorable novel biomarker for the prognosis of BC and CaP patients.

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Cytotoxic and photodynamic activity of new cationic porphyrins

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Among porphyrins used in the photodynamic therapy of tumors (PDT), special place is occupied cationic porphyrins, due to their higher selectivity of accumulation in malignant cells than the anionic and neutral [1]. Previously we synthesized a set of watersoluble cationic porphyrins (PSs) with different peripheral functional groups and metalloporphyrins with different central metal atoms [2]. For PDT and photodynamic inactivation of microorganisms (PDI) we were tested new cationic porphyrins with different charges and hydrocarbon ‘tails’. Cancer cell culture (monolayer and suspension) equally well destroyed by cytotoxic or phototoxic action of new cationic porphyrins with charge +3 and +4. By photodynamic effect on culture of cells K-562 (chronic myelogenous leukemia lymphoblasts) the most effective preparations are Zn-metalloporphyrins with hydrocarbon ‘tails’ (C12 or -C16). Photodynamic actions of metalloporphyrins are in 10–20 times more effective than the cytotoxic action the same porphyrins. It is established that in vitro for the destruction of Gram (+) and Gram (-) microorganisms in photodynamic mode cationic water-soluble synthetic metalloporphyrins, especially Zn-meso-tetra[4-N-(2'-butyl)pyridyl] porphyrin (Zn-TBut4PyP), many times more effective than Zn-porphyrins, synthesized from the nature origin. In vivo conditions on mice established that the best therapeutic activity against various strains of the microorganism S. aureus has the synthetic metalloporphyrin Ag(TBut4PyP). Thus new cationic porphyrins are highly active and can be recommended for further testing for application in PDT and in PDI.

References


Abstracts

SW04.S16–152
Inhibition of poly(ADP-ribose) polymerases partially reduces the cytotoxic effect of doxorubicin on cultured rat cardiomyocytes
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Doxorubicin (Dox) is an anthracycline antineoplastic agent widely utilized in cancer therapy. However, cardiotoxicity is an unpleasant side effect of doxorubicin, leading to serious cardiovascular complications and increased mortality in people undergoing treatment with this drug. Different assumptions about the possible causes of cardiotoxicity of doxorubicin were made early, and one of them based on the fact that doxorubicin can cause the development of oxidative and nitrosative stresses.

**Purpose:** To ascertain whether the inhibition of the poly(ADP-ribose) polymerases (PARPs), an enzymes involved in cell response to oxidative stress, affect the cytotoxic properties of doxorubicin in experiments with cultured cardiomyocytes.

**Methods:** The line of rat cardiomyoblasts H9c2 and primary cardiomyocytes from neonatal rats were used in our study. The cell survival was estimated by using convenient MTT-test (cell staining with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The percent of apoptotic or necrotic cells was evaluated by double staining with propidim iodide and Hoechst 33258.

**Results:** Long-time (24, 48 and 72 hours) incubation of both, H9c2 cells and primary cardiomyocytes, with Dox (0.5–10 μM) caused a marked concentration-dependent cell death. PARPs inhibitors (3-aminobenzamide or PJ-34) significantly increased cell survival, reduced the percent of apoptotic cells in both, H9c2 cells and primary cardiomyocytes, and restored the proliferative capacity of H9c2 cells compared to cultures treated only with Dox. Interestingly the cytoprotective effects of PARPs inhibitors were observed only in narrow range of Dox concentrations – from 1 to 2 μM.

**Conclusions:** In summary we have shown that PARP inhibitors decrease Dox toxicity toward H9c2 cells and primary cardiomyocytes and can be considered as potential cardioprotective agents at the Dox cancer therapy.

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SW04.S16–153
Comparative analysis of proanthocyanidins protective effects in cytostatic-treated normal and malignant cells
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Antitumor drugs doxorubicin (DOX) and mitomycin C (MMC) exert the cytotoxic effects in malignant cells, but also induce the generation of reactive oxygen species (ROS) and oxidative damage in normal cells and tissues during chemotherapy. The main objective of our study was to investigate whether proanthocyanidins (PAC) have the capacity to modulate the levels of antioxidative system activities, particularly of glutathione (GSH) and GSH-dependent enzymes, in both normal and malignant cells. Possible cytoprotective effects of PAC and their influence on modulation of antioxidant status of cells in our study were compared with the effects of known cytoprotective agent – N-acetyl cysteine (NAC).

The parameters of oxidant status – activity of antioxidant enzymes glutathione-S-transferase (GST) and glutathione reductase (GR) were tested in human erythroleukemia (K562) and chinese hamster ovary (CHO) cell lines. Both cell lines were pre-treated with potentially protective agents (NAC, PAC), 30 min before DOX and MMC, as pro-oxidant agents. Cell supernatants were prepared after 24 hours and GST and GR, as parameters of oxidant status, were determined.

Results of our study suggest that proanthocyanidins and N-acetyl cysteine exert the antioxidative activity and therefore the potentially protective activity to the effects of doxorubicin and mitomycin C. Comparative analysis of activities of antioxidant enzymes GR and GST in malignant and normal cell lines shows that none of those two antioxidant agents expresses selective activity to non-malignant, CHO cells.

Our results indicate the complexity of biological response on PAC-induced effects. Further investigation is needed to get more detailed insight into mechanisms of antioxidant defense, mediated by PAC, in normal cells.

SW04.S16–154
Fusion proteins capable of selective binding to melanoma cells for investigation of the mechanisms of cell malignization
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Metastasis is multiple-stage process, which is characterized by a number of rounds of metastasizing cell attachment/detachment to extracellular matrix. Different cell adhesion molecules such as cadherins and integrins take part in this process. More over the proper cooperation of different molecular structures is required. Integrin receptors are able to take part in the regulation of such cooperation because of its ability to bidirectional signal transduction and the mediation of cell adhesion, migration, proliferation, differentiation and apoptosis [1]. Observations showing that integrin overexpression correlates with the increase of the metastatic potential of melanoma cells [2] suggest the key role of integrins in melanoma cell malignization.

It is known that integrins which are overexpressed in highly-metastatic melanoma cells are capable to high affinity binding to RGD tripeptide, which has been found in a wide variety of its natural ligands [3]. So RGD-bearing proteins having the ability to selective
binding with melanoma cells represent the suitable tool for investigation of the mechanisms of melanoma cell malignization.

In present work expression vectors bearing the genes of fusion proteins have been constructed for producing these proteins in E. coli. Such fusion proteins consist of a peptide ‘address’, targeting the integrins on melanoma cells, linked to an ‘adaptor’ for the attachment of a visualizing agent. The peptide ‘address’ contains the RGD motif, which is stabilized by a disulfide bond to achieve the optimal receptor binding conformation. The ‘adaptor’ is a tetrameric protein, namely, streptavidin, that is able to achieve high-affinity binding of d-biotin, which facilitates the generation of colored proteins using the appropriate biotin derivatives. Aforementioned proteins were purified from the periplasm of E. coli using columns with 2-iminobiotin agarose and demonstrated ability to selective adhesion to murine and human melanoma cells.

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References

SW04.S16–155
A single mechanism of cancer for all carcinogenic factors
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There are many factors which action on organism could lead to cancer. They are physical, chemical, mechanical, biological (bacteria, viruses or some genetic material) but all of them has a single mechanism of carcinogenesis. Its name – the energy-related model of carcinogenesis [1,2].

Process of transformation of normal cells of a live organism in the cancer ones was considered from power positions. It is shown that change of genetic properties of cells, connected with change of its molecular structure – or with changes of DNA bases, occurring without its destruction. It is possible only at external influences of quanta energies of 4–8 eV. Such influences are corresponded to ultra-violet (UV) to range.

It is shown that under the influence of radiation UV in DNA could change the molecular structure of DNA bases.

If such changes are happen in protooncogenes sites, they can lead to their, with some probability. Transformation into active oncogenes.

It is shown by complex international researches. That UV radiation possess carcinogenic properties.

The analysis of an action of all other carcinogenic factors.

On an organism is showed that all of them are also concentrated in UV spectral range.

Except direct action on cell genome UV radiation are capable to weaken reparation abilities of cells and immunity of an organism. Thus, at long action of UV radiation are create conditions for save these changes in cell genome and the transformed cancer cells have created.

The offered mechanism of carcinogenesis allowed to explain features of emergence and development of cancer diseases both natural, and experimental character.

The offered mechanism of carcinogenesis allowed explaining features of cancer at adult persons, at the children and at the embryos of men.

With its help safety of application of low intensive therapeutic laser influences was theoretically proved.

References

SW04.S16–156
Effect of Schiff base combination on liver cancer
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Schiff bases are condensation of aldehyde or a ketone with primary amine groups under specific conditions and they contains C=N groups in they structure. Schiff base has a nitrogen analogue of an aldehyde or ketone in which the carbonyl group (C=O) has been replaced by an imine or azomethine group as structurally. Schiff bases can be used in reduction or oxidation reactions as catalyast. Furthermore, they have some biological activities such as anticancer, antifungal, antibacterial, anti-inflammatory and antiviral. (1,2,3) In this study, we have tested some derivatives of Schiff bases on Hep3B (liver cancer cell line) cell line in different concentrations for 3 days with 24 hours intervals. For control group, dental follicle cell line was used. As a in vivo experimental model, aoxymethane and DNA induced liver cancer model was used on rats. Our results shows that Schiff base-derivatives in combination with block copolymers were effective on Hep3B cell line reducing cell viability. Dental stem cells used as control cell line, showed less toxicity when compared to Hep3B cell line. Rats treated with low concentrations of Schiff base combinations demonstrated no toxicity and failed to develop liver cancer inspite of aoxymethane and DNA treatment. These data indicates that Schiff base combination with block copolymers could serve as anti-cancer agents for the treatment of liver cancer cells.

References

SW04.S16–157
Pre-clinical trials of the new antiCDK4/6 MMD37K peptide
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Background: MMD37K is synthetic peptide which include leading sequence -CPP-(Antp, Penetratin) and cyclone-dependent kinase inhibition motive from p16INK4a with Mv~4000 D. We investigated in vitro and in vivo cytotoxic, cytostatic and antitumor activity of MMD37K. Full study of Toxicology tests were produced.

Methods: Cytotoxic effects were investigated on different cell line (Jurcat, Raji, A549, MCF-7, Hct-116, Ht-29, HEK293) in
the range of concentration 0.1–100 mM MMD37K for 24–48 hours. Proliferation (MTT), DNA-content, cell cycle (flow cytometry) and levels of mRNA cyclin A, Kif67, bax, bcl-2 and pRb phosphorylation were investigated. In vivo experiments were provided on xenograft model of HCT116, A-549 at concentration 5 and 10 mg/kg. Toxicology experiments were provided according RF Low and include three types of animals. LC-MS method of detection MMD37K in plasma was work out.

**Results:** Our results proved that MMD37K stop G1-S transition and prevented pRb phosphorylation and proliferation activation in all investigated cell line. Cytostatic effect did not depend on p16 mutation or expression. MMD37K induced apoptosis in all investigated cells lines with IC50 = 30–40 mM. Antitumor effects in xenograft model were 53% for A-549 and 67% for HCT116. Toxicological studies have shown that LD10 for rats is 100 mg/kg and no dieds were registered for rabbits. Pharmacokinetics had two phases: for 1st – t1/2 = 2.39 ± 0.39 min and for 2nd t1/2 = 2 – 2.39 ± 0.39 hours.

**Conclusion:** Our investigations showed stable cytostatic and cytotoxic antitumor effect of MMD37K in investigated cell line in vitro and in vivo. Toxicological and pharmacokinetics results allow us to hope for good tolerability MMD37K in human and recommended to start clinical investigations.

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**SW04.S16–158**

**Cell penetrating peptides (CPP) as the intracellular delivery system for anticancer agents**

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Although chemotherapy has been responsible for curing many people of cancer in the first decade of the 21st century, there still remain a large number of patients whose tumors still are not curable. There has been substantial investment in researching the mechanisms used by normal eukaryotic cells to control progress through the cell cycle in the hope that this would lead to an understanding of how cancer arises and suggest possible targets for cancer therapy.

During carcinogenesis, it is currently thought that normal cells become immortalised as a consequence of disruption of the positive and negative cell signalling pathways and cell cycle control mechanisms, for example, amplification and overexpression of cyclins and CDKs. Amplification and overexpression of cyclin D protein occurs in many human tumors.

We investigate the ability to direct the import of therapeutic agents into cells and target them to specific organelles with specific cell penetrating peptides (CPP). Transduction of protein of interest into cells by the help of peptides can serve as a good alternative to other mechanism of protein transport and protein expression. So we can conclude CPP only possible way for effective intracellular delivery this new therapeutic agents.

We investigate transport properties a wide range of peptides with different sequences and position of leading CPP in different part of chimeric peptide molecules. Based on our results we proposed new mathematical model for intersection of the cell membrane by CPP.

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**SW04.S16–159**

**In vitro and cellular study of benzothiophene-3-carboxamides as inhibitors of Aurora kinase family**

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Serine/threonine kinases Aurora A, B and C play essential role in mitotic process, cytokinesis and maintaining the genomic integrity of normal cells. Aurora A and B kinases are often overexpressed in various tumor types and their inhibition results in mitotic arrest and eventually in apoptosis of the tumor cells. Up till now several Aurora inhibitor agents are in clinical development for the treatment of solid and blood cancers.

Applying in vitro recombinant kinase assay we identified novel benzothiophene-3-carboxamide derivatives as potent Aurora A and B kinase inhibitors in the molecular library of Vichem Chemie Ltd. Whether these compounds inhibit cell cycle and induce apoptosis on tumor cells, we compared them to clinical aurora kinase inhibitors in further recombinant kinase and cell-based assays.

For in vitro tests we utilized the IMAP fluorescent recombinant kinase assay. The antiproliferative effects were screened on colon carcinoma cell lines (Aurora overexpressing HCT-116 and HCT-15 with normal expression level of Aurora kinases) using the MTI assay. Mitotic arrest and induction of early and late apoptosis were assessed by flow cytometry on the same cell lines. Inhibition of Aurora A and B kinase activity was proved by SDS-PAGE and immunoblotting.

Most of the compounds tested inhibited either Aurora A or B kinases in vitro. However only a subset had antiproliferative effect on cell lines. These selected compounds indeed caused G2 arrest, and inhibited cytokinesis, what resulted in polyploid, multinucleated cells. Moderately elevated level of apoptotic cells occurred only after longer incubation with these compounds. However, all results were comparable to the effect of the reference compounds.

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**SW04.S16–160**

**Therapeutic enzymes for the treatment of leukemia: Autoproteolytic activation of human asparaginase induced by free glycine**

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Asparaginases catalyze the deamination of the amino acid asparagine to aspartate and ammonia. Bacterial asparaginases are used in cancer chemotherapy to deplete blood asparagine levels, since several hematological malignancies depend for growth on the extracellular supply of the non-essential amino acid asparagine. To avoid the immune response and other side effects against the therapeutically used bacterial enzymes, it would be beneficial to replace them with human asparaginases. The human genome codes for at least three enzymes with asparaginase activity. One of them, which we refer to as hASNase3, belongs to the N-terminal nucleophile (Ntn) hydrolase superfamily where the protein is made as a single polypeptide that is devoid of catalytic activity. Self-cleavage releases the amino group of Thr168, a moiety required for catalyzing asparagine hydrolysis. Increased expres-
Chemotherapeutic and radiotherapeutic treatments induce overexpression of the multidrug resistance 1 (MDR1) in gallbladder cancer cell models

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In Chile, the gallbladder cancer (GBC) is the leading cause of death for neoplasms in women over 40 years. This tumor is considered highly resistant to the chemotherapy. The overexpression of multidrug resistance protein MDR1 is an important mechanism of chemo-resistance in many kinds of neoplasms, but studies on gallbladder cancer are scarce.

The aim of this work was to determine if current treatments for gallbladder cancer induce the expression of drug resistance protein MDR1.

As biological model of GBC was used the cell lines G415 and Gbd1. IC50 values after chemotherapeutic treatments with 5-fluorouracil, cisplatin, gemcitabine and paclitaxel was performed by MTT assay. Cell survival status was performed after different doses of ionizing radiation through the clonogenic assay. The levels of MDR1 after chemo-radiotherapeutical treatments were performed through qRT-PCR and Western blot. The expression of DNA methyltransferase-1 (DNMT1) was determined by Western Blot.

The chemo-radiotherapeutical treatments produce an increase in the expression of MDR1 in the cell line Gbd1 but no expression in G-415. In other hand the treatments produced a decrease in the expression of DNMT1.

We conclude that chemo-radiotherapeutical treatments currently used against gallbladder cancer could induce chemo-resistance where MDR1 could play a pivotal role in resistance to chemotherapeutic agents. Besides, we give evidence that the expression of MDR1 could be correlated with the methylation state of its gene promoter.

This work was supported by Grants 1120903 and 15110027 from FONDECYT, and FONDAP, respectively, Conicyt, CHILE.
ALT ratio >1 demonstrated higher MMP-9 serum levels than those with ratio <1 (p < 0.001). The same was observed for caspase-3; however, it did not reach statistical significance. A significant correlation was observed between these markers and AST as well as AST/ALT ratio. However, no correlation was observed with the viral load. In conclusion, MMP-9 and caspase-3 might be involved in HCV-associated liver injury. Measurement of MMP-9 and caspase-3 levels could be reliable markers for liver damage, which may open up new diagnostic and therapeutic strategies aimed at manipulating the early inflammation as well as the late fibrotic changes in HCV infection.

**SW04.S16–164**

**Gene polymorphisms of matrix metalloproteinase-9 in chronic and aggressive periodontitis in Turkish population**

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Periodontal disease is the main disease of the oral cavity and the cause tooth loss in human. Periodontitis is a widespread destructive inflammatory process affecting the tooth-supporting tissues including gingiva, cementum, alveolar bone and periodontal ligament. Research intended for genetic markers associated with the susceptibility and severity for periodontal disease is receiving considerable attention in the last decades. Matrix metalloproteinases (MMPs) are thought to relate tissue destruction and remodelling in periodontal diseases. Metalloproteinases are a family of Zn-dependent endopeptidases that degrade the extracellular matrix. These enzymes are implied in the pathologic processes of the oral cavity as the destruction of the periodontal tissues. The MMP-9 also has a gelatin-binding domain, inserted between the catalytic and the active domains for which the MMP-9 is also known as gelatinase B. Increased expression of MMP-9 was found in the periodontitis sites, decreasing after the treatment and higher MMP-9 levels in the gingival crevicular fluid was reported to be associated with the severity of the disease. The aim of this study was to analyze MMP-9 (-1562) C/T polymorphisms in chronic periodontitis (CP) and aggressive periodontitis (AP) patients in a Turkish population.

CP, AP and periodontally healthy individuals were included in the study. All participants had no systemic diseases. After clinical and radiographic examinations, blood samples were obtained and genomic DNA was extracted from peripheral blood using QIAamp Blood MiniKit according to the manufacturer’s instructions. Single nucleotide polymorphisms (SNP) were analyzed by PCR (Polymerase Chain Reaction) and RFLP (Restriction Fragment Length Polymorphism) methods in DNA samples. For MMP-9 (-1562) C/T polymorphism; CC, TT and CT genotype and allele frequencies were analyzed in CP and AP patients and controls.

The preliminary findings of this study indicated significant differences in genotype and allele frequencies between the groups with respect to SNPs in MMP-9 genes in Turkish population. Further studies including greater number of subjects and performing linkage analyses may provide more supporting results.

**References**


**SW04.S16–165**

**(R)-, (S)- and (R,S)-Phenyl tridecyl carbinols exhibit antielastase and anticollagenase activities**

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Chiral alcohols are valuable substances for the food, drug and cosmetic industries because of their biological activity which can depend on their enantiomeric and racemic forms. The present study deals with the analyse of the antielastase and anticollagenase activities of the optically pure R and S enantiomers of chiral and racemic phenyl tridecyl carbinols. Until now, there has been no report on the antielastase and anticollagenase activities of chiral and racemic phenyl tridecyl carbinols.

Chiral phenyl tridecyl carbinol was synthesized by different asymmetric reduction methods from their corresponding ketone [1] and by enzymatic resolution of its racemic carbinol by Yildiz and Yusufoglu [2]. All the test compounds exhibited antielastase and anticollagenase activities. The inhibition was increased with increasing phenyl tridecyl carbinol concentration. Lower IC50 values indicate higher enzyme inhibitory activity.

A high elastase inhibition (87.41 ± 2.18%) was seen in 0.1 mg/ml for (R)-phenyl tridecyl carbinol and IC50 value was found 0.068 µg/ml. The compound (R)-proved to be the most potent showing a collagenase inhibition activity with an IC50 = 0.00056 mg/ml.

The results showed that (R)-phenyl tridecyl carbinol had effective antielastase and anticollagenase activities. These compounds could be used as a source of antielastase and anticollagenase compounds in cosmetic and pharmaceutical industries.

**References**


**SW04.S16–166**

**Antiurease, antielastase and antioxidant activities of some monohydroxy tetradecanoic acid isomers**

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Hydroxy fatty acids are ubiquitous in nature and have been found as constituents of triacylglycerols, waxes, cerebrosides, and other lipids in plants, animals, insects and microorganisms [1,2]. The hydroxy fatty acids mentioned in the literature are valuable starting compounds used in the preparation of numerous textile auxiliaries, detergent, dispersion and emulsion reagents [3] and play an important role in cancer chemotherapy [4]. Hydroxy fatty acids exhibited antitumor effect in neutral and acidic pH values [5,6]. In the literature, there is no data on the antiurease, antielastase, and antioxidant activities of monohydroxy tetradecanoic acid isomers. In this study, antiurease, antielastase and antioxidant activities of 3,6,7,9,12- monohydroxy tetradecanoic acid isomers were determined for the first time. All the test isomers, which have been synthesized previously [7] by Hasdemir et al., exhibited antiurease, antielastase and antioxidant activities. The enzyme inhibitory and
antioxidant activities of these mentioned isomers were found to increase dose dependently. As another result, these activities showed different inhibition and antioxidant activities according to the position of the hydroxy group on the carbon chain length.

These mentioned 3,6,7,9,12-monohydroxy tetradecanoic acid isomers can be used in agriculture, pharmacy and cosmetic industries due to their excellent antiiurease, antielastase and antioxidant activities, tested in this study.

References

SW04.S16–167
Antielastase and antityrosinase activities of racemic and chiral phenyl dodecyl carbinols
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Enantiomerically pure or enriched carbinols are of very high importance as intermediates for the synthesis of a wide variety of targets such as pharmaceuticals, pesticides, food and cosmetics, and so on. According to the literature no other study has examined antielastase and antityrosinase activities of racemic and chiral phenyl dodecyl carbinols. The aim of this study is to investigate antielastase and antityrosinase activities of racemic and chiral phenyl dodecyl carbinols which has been synthesized previously by Yildiz and Yusufoglu [1,2].

All (R,S)-(R)- and (S)-carbinols exhibit antielastase and antityrosinase activities. (R)-Phenyl dodecyl carbinol showed the highest elastase inhibition activity (IC50 = 0.0095 mg/ml). The activity of (S)-enantiomer (IC50 = 0.022 mg/ml) was higher than the racemic (R,S)-carbinol (IC50 = 0.0322 mg/ml).

The highest tyrosinase inhibition activity was determined for the racemic (R,S)-carbinol (IC50 = 0.00010 mg/ml). (R)-Enantiomer (IC50 = 0.00039 mg/ml) was more effective than the (S)-enantiomer (IC50 = 0.00047 mg/ml). All these compounds exhibit antielastase, antityrosinase activities due to their concentration. The collagenase inhibition activity is decreasing as RS>R>S. The highest antielastase activity is found for R enantiomer, S is more active than the racemic RS. The tyrosinase inhibition activity exhibited the following order: RS>S>R.

According to these results, racemic form and optically chiral R and S enantiomers of 3-hydroxytetradecanoic acid may be considered as an important source of pharmaceutical and cosmetic area due to their collagenase, elastase and tyrosinase inhibition activities.

References

SW04.S16–168
Effect of chirality of 3-hydroxy tetradecanoic acid enantiomers on skin diseases related enzymes
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β-Hydroxy acids are gaining great importance since they can be useful synths for synthesis of pharmaceuticals, vitamins, flavours, antibiotics and phenomers [1]. Optically active (R)-3-hydroxytetradecanoic acid is the most common fatty acid constituent of lipid A. The lipid A analogue with the S configuration was more strongly bioactive than the natural R type [2]. R-β-Hydroxy alkanolic acids represent an important class of biologically active compounds often found in lipopeptides exhibiting antimicrobial, insecticidal and antiviral activities [3]. Skin is the largest organ of the human. It produces free radicals or reactive oxygen species due to repeated sun exposure, which leads to oxidative stresses and inflammatory responses in the dermal or epidermal layer of the connective tissues resulting aging and damage of cell membranes, lipids, proteins and DNA. In recent years collagenase, elastase, and tyrosinase inhibition have gained great importance in skin disease. According to the literature no other study has examined the anticollagenase, antielastase and antityrosinase activities of chiral 3-hydroxytetradecanoic acids. The aim of this study is to analyse the anticollagenase, antielastase and antityrosinase activities of racemic form and optically pure R and S enantiomers of 3-hydroxytetradecanoic acid which have been synthesized previously by Kucuk and Yusufoglu [4].

All these compounds exhibit anticollagenase, antielastase and antityrosinase activities due to their concentration. The collagenase inhibition activity is decreasing as RS>R>S. The highest antielastase activity is found for R enantiomer, S is more active than the racemic RS. The tyrosinase inhibition activity exhibited the following order: RS>S>R.

According to these results, racemic form and optically chiral R and S enantiomers of 3-hydroxytetradecanoic acid may be considered as an important source of pharmaceutical and cosmetic area due to their collagenase, elastase and tyrosinase inhibition activities.

References
SW04.S16–169
Stability of Lysobacter sp. XL1 AlpA and AlpB endopeptidase complexes of their pro regions and mature enzymes
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Lytic enzymes represent an alternative attractive to antibiotics in treatment of infections caused by multidrug-resistant microorganisms. In our research we have investigated the lytic endopeptidases AlpA and AlpB from Lysobacter sp. XL1. These enzymes belong to the class of serine proteases that are homologues of \( \alpha - \)lytic protease of \( L. \)~\( \text{ex} \text{zymogen} \)es and have a wide range of antimicrobial activity different from each other. As well as \( \alpha - \)lytic protease, AlpA and AlpB are expressed as precursor protein, which consists of the leader peptide, the pro region and the mature enzyme. Pro region has a dual function: it provides the correct folding of the enzyme and inhibits its activity. The complex between the pro region and mature enzyme is formed by the interaction in several sites, including the active center of the enzyme. We have studied the formation of a complex between the pro regions and mature endopeptidases of AlpA and AlpB. It was found that mature AlpA formed a stable complex with its pro region, and an exogenous proteolytic cleavage of pro region was necessary for the activation of endopeptidase. Unlike AlpA, AlpB has shown the ability for self-activation, which can lead to the loss of activity of the enzyme preparation during its long-term storage. Based on the analysis of AlpB primary sequence and tertiary structure models, we have constructed a chimeric pro region, and assessed its ability to provide the correct folding of the mature enzyme and to inhibit its activity. We have found that the chimeric pro region in cis (fused to the mature enzyme) was not able to provide the folding of the endopeptidase. At the same time, the chimeric pro region added in trans, formed a stable complex with mature AlpB which had a long half-life.

SW04.S16–170
Some 3–13 monohydroxy eicosanoic acid isomers exhibit antielastase, antiiurease and antioxidant activities
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Hydroxy fatty acids are long-chain fatty acids with one or more hydroxyl groups in their chemical structures. They are widely used as surfactants, lubricants, cosmetics, antimicrobials, pharmaceutical intermediates, and synthetic precursors in polymer chemistry [1]. A series of some 3–13 monohydroxy eicosanoic acid isomers were evaluated for their antielastase, antiiurease and antioxidant activities in this study. Some 3–13 hydroxy eicosanoic acids have been synthesized with high purity from Celik et al. [2,3]. All the test compounds exhibited antielastase, antiiurease and antioxidant activities in this study. The enzyme inhibitory and antioxidant activities of these isomers were found to increase dose dependently. According to the obtained results the hydroxy eicosanoic acid isomers locating in the middle and close to the middle of the chain showed higher antielastase, antiiurease and antioxidant activities rather than that of the other isomers. Therefore, 3–13 monohydroxy eicosanoic acid isomers can be used in agriculture, food additives, pharmacy and cosmetic industries due to their excellent antielastase, antiiurease and antioxidant activities.

References

SW04.S171–170
The lysozyme sustained release system based on poly(3-hydroxybutyrate)-poly(ethylene glycol) microparticles
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Development and biochemical study of proteins sustained release systems is a promising trend in modern biopharmacology. Application of these devices can eliminate the most of the drawbacks of traditional medicines: high toxicity, infectivity, substance instability, inconvenience of administration etc. The biodegradable polymer poly(3-hydroxybutyrate) (PHB) and its copolymers, obtained biotechnologically in our laboratory with Azotobacter chroococcum strain 7B, are used to develop a wide range of devices for biomedical applications including proteins sustained release systems. Here we investigated microparticles loaded with lysozyme that were produced by two-step emulsification solid/ol/water (S/O/W) technique. For microparticles production we used a novel PHB copolymer poly(3-hydroxybutyrate)-poly(ethylene glycol) (PEG-PHB) [1] with molecular weight of 255000, as well as PHB with molecular weights of 25 000 and 255 000 for comparison. We have chosen lysozyme as model protein with enzymatic activity. A lysozyme sustained release system from biopolymer microparticles was carried out in vitro in a phosphate buffer (pH 7,4) at 37°C. We showed that protein release from the microparticles occurs via two processes: diffusion and degradation of microparticles. The release kinetics of the protein was connected with polymer molecular weight and hydrophobicity. Therefore, to improve the performance of lysozyme sustained release we used a more hydrophile PHB-PEG. As a result, the release kinetics of PHB-PEG microparticles was significantly more linear than that of PHB microparticles. The efficiency of protein encapsulating to PHB-PEG microparticles was also better. However, the encapsulated protein can lose its native structure and enzymatic activity during its release from polymer microparticles. To verify lysozyme integrity we carried out the SDS-electrophoresis of released protein and examined the lysozyme enzymatic activity. The obtained data demonstrated that the lysozyme do not lose their integrity and enzymatic activity for 14 days sustained release from microparticles. Thus, the developed technique for protein encapsulation in PHB-PEG microparticles can be used for development of various therapeutic protein sustained release systems.

Reference
**SW04.S16–172**

**Candidate breast cancer DNA vaccine: design of polyepitope antigen and evaluation of its expression in human dendritic cells**

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Breast cancer is one of the most common malignancies in women and is the second leading cause of cancer death. In the modern era of breast cancer treatment, immunotherapy has emerged as an effective tool for improving clinical outcomes. We propose to induce breast-cancer-specific CD8⁺ T cell response after transfection of dendritic cells with synthetic polyepitope DNA vaccine. Polyepitope antigen (PA) encoding DNA sequence has been designed from HLA-A*0201 restricted CTL epitopes previously described and predicted with TEPredict software in the structure of two tumor-specific breast cancer antigens – HER2 and Mammoglobin 1. PolyCTLDesigner software was used for optimization of target antigen structure including proteolytic sites localization signals and Gag-epitope for monitoring expression of target antigen.

*Candidate breast cancer DNA vaccine: design of polyepitope antigen and evaluation of its expression in human dendritic cells*  
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Breast cancer is one of the most common malignancies in women and is the second leading cause of cancer death. In the modern era of breast cancer treatment, immunotherapy has emerged as an effective tool for improving clinical outcomes. We propose to induce breast-cancer-specific CD8⁺ T cell response after transfection of dendritic cells with synthetic polyepitope DNA vaccine. Polyepitope antigen (PA) encoding DNA sequence has been designed from HLA-A*0201 restricted CTL epitopes previously described and predicted with TEPredict software in the structure of two tumor-specific breast cancer antigens – HER2 and Mammoglobin 1. PolyCTLDesigner software was used for optimization of target antigen structure including proteolytic sites localization signals and Gag-epitope for monitoring expression of target antigen.

**SW04.S16–173**

**Programmed tumor cells death induced by recombinant analog of lactaptin**

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Previously we have found the proteins of human milk which are capable to induce the apoptotic death of human mammary adenocarcinoma cells MCF-7. Lactaptin – one of the pro-apoptotic proteins of human milk – was purified, characterized and identified as proteolytic fragment of kappa-casein [1]. Subsequently we constructed the recombinant analog of lactaptin (RL2) which induced apoptosis of MCF-7 cells [2]. Apoptosis of MCF-7 was characterized by activation of pro-caspase 7 leading to accumulation of the enzyme active form [3].

We have shown that apoptotic death of RL2 treated MCF-7 cells accompanied by phosphatidylinerse exposure on the plasma membrane and activation of caspase 7. At that activation of DNAase DFF40 and oligonucleosomal fragmentation of DNA didn’t take place [2].

The analysis of biochemical markers of RL2 induced MCF-7 apoptosis was the main task of our investigation. To analyze the mitochondrial inner membrane permeability we have measured mitochondrial transmembrane potential (ΔΨ) of MCF-7. It was shown that RL2 induced dissipation of ΔΨ of MCF-7 cells.

To investigate the apoptotic cascades (intrinsic or extrinsic) triggered by RL2 we performed analysis of initiator caspase 8 and caspase 9 activation. It was shown that RL2 induced the accumulation of both caspases 8 and 9 active forms. Thus we can suppose that the elements of intrinsic and extrinsic apoptotic pathway are involved in apoptotic death of the cells.

In this work we also have performed whole-genome HT assay for expression profiling of RL2 treated MCF-7 cells (Illumina). We have shown RL2 initiated the consecutive increase (8, 18, 28 hours) of mRNAs levels for the following gene groups: condensed chromosome kinetochore, microtubule cytoskeleton organization, regulation of mitotic cell cycle, cell cycle arrest. Moreover increasing level of p53 mRNAs as well as mRNAs of p53 upregulated MCF-7 genes were observed.

Identification of human MCF-7 cells proteins interacting with RL2 was accomplished by affinity purification coupled with tandem mass spectrometry method. We identified three proteins of cytoskeleton interacted with RL2: α and β chains of tubulin and α-actinin-1. Tubulin forms heterodimers and assembles into microtubules; α-actinin-1 is a homodimer that cross-links actin filaments into parallel bundles.

It is well known that cell cycle arrest at G2/M phase can occurs when microtubules dynamic of mitotic spindle is suppressed. During G2/M arrest cells can die through caspase-dependent or caspase-independent apoptosis upregulated by p53 [4]. Overexpression of p53 can lead to mitochondrial membrane permeabilization, loss of ΔΨ and activation of initiative caspase 9 [5]. Summarize, we have proposed the molecular mechanism of tumor cell death induced by recombinant analog of lactaptin.

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**References**


**SW04.S16–174**

**Towards a new understanding of the bacterial cell wall**

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The bacterial cell wall has long been recognised as a key site of action for antibiotics which function through exploiting the dif-
ferential recognition of the machinery responsible for its maintenance compared to that in man. Despite its importance for the development of new medicines, the structure of this important target remains shrouded in mystery and the cell wall persists as one of the major building blocks of life where little progress in understanding its architecture has been made over the last 50 years. The lecture will describe crystallographic studies on components of the bacterial cell wall which have provided new insights into the structure and organisation of the bacterial peptidoglycan layer leading to a new model for its arrangement with profound implications for the discovery of novel anti bacterial agents.

**SW04.S16–175**

Antimalarial activity of cupredoxins: The interaction of *Plasmodium Merozoite Surface Protein 1* (MSP1<sub>19</sub>) and Rusticyanin

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Finding effective new antimalarial agents is an urgent need. One of the most studied molecules anchored on the parasite surface is the C-terminal fragment of the Merozoite Surface Protein 1 (MSP1<sub>19</sub>), which is taken into the red blood cell at invasion and remains intact in the digestive food vacuole to the end of the intracellular cycle. Thus, MSP1<sub>19</sub> is a promising target against malaria since a number of specific antibodies inhibit erythrocyte invasion and parasite growth. Given the structural homology of cupredoxins family with F<sub>0</sub> domain of monoclonal antibodies and previous evidences of parasitemia inhibition due to the interaction of azurin with MSP1<sub>19</sub>, an approach combining NMR and ITC measurements with BiGGER-based docking calculations is performed on MSP1<sub>19</sub>/cupredoxins complexes. Among tested cupredoxins, rusticyanin forms a well-defined complex with MSP1<sub>19</sub> in a site that overlaps with the surface recognized by the inhibitory antibodies. These findings become rusticyanin as an excellent therapeutic tool for malaria treatment and provide valuable information in drug design.

**SW04.S16–176**

Nicotinic acetylcholine receptors in mitochondria: New role for the old player

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Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels mediating fast synaptic transmission in nerve and muscle cells. They are also expressed in many non-excitable cells to regulate vital cellular processes like proliferation, adhesion and survival, as well as production of cytokines. We have found for the first time that functional nAChRs of neuronal type are present in mitochondria outer membrane. This was shown by electron and fluorescent confocal microscopy and confirmed by multiple assays using nAChR subunit-specific antibodies and toxins in isolated mitochondria of mouse liver, brain and lung, as well as in human cell lines of various origins. The nAChRs containing α7 subunits were found to be coupled to voltage-dependent anion channels of mitochondria outer membrane and to regulate Ca<sup>2+</sup> accumulation and early apoptotic events like superoxide and cytochrome c release stimulated by either Ca<sup>2+</sup> or H<sub>2</sub>O<sub>2</sub>. Interestingly, the mechanism of mitochondrial α7 nAChR functioning appeared to be ion channel-independent since attenuation of cytochrome c release could be achieved by the binding of specific agonists (acetylcholine, choline, PNU 282987), antagonists (methyllycaconitine, hexamethonium), allosteric modulator PNU 120526 or antibodies against extracellular epitopes of α7 nAChR subunit. By using specific kinase inhibitors and antibodies, it was found that α7 nAChRs regulating activity involved inhibition of intramitochondrial CaMKII, Src and PKC pathway(s) and activation of PI3K/Akt pathway. These data allow concluding that, in addition to established anti-apoptotic signaling pathways, there is an intracellular, previously unrecognized cholinergic mechanism to control mitochondria apoptotic susceptibility. Probably, it belongs to the most ancient survival mechanisms inherited by mitochondria from their hypothetic prokaryotic ancestor. This finding offers a novel view on the mitochondria protection in apoptosis and opens the way for its pharmacological regulation.

**SW04.S16–177**

Conjugate of human oxyntomodulin and polysialic acid has a prolonged anorexic effect in vivo

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Recombinant gut hormone oxyntomodulin (OXM) is known to act as a satiety signal in human subjects and has therapeutic potential as an appetite controlling agent. The only form of this hormone that has a prospective use is a modified one, because native OXM has a very short half-life in vivo. Conjugation of OXM and the natural hydrophilic polymer polysialic acid (PSA) may significantly improve its half-life. Chemical polsialylation in vitro was used to create a long-acting form of OXM, the polysialic acid-oxyntomodulin (PSA-OXM) conjugate. The single conjugation site was identified on N-terminus using mass shift comparative analysis of Asp-N proteolytic digests. A two-stage purification technique was developed to obtain a homogeneous PSA-OXM conjugate, suitable for in vivo testing.

The conjugate obtained was resistant to the DPP-IV protease. A single injection of PSAeOXM at 15 mkmol/kg dose was sufficient to maintain a steady decrease in food consumption for 8 h (p < 0.05) on the lean, fasted mouse model. The length of the anorexic effect achieved is comparable to other long-acting derivatives of OXM but it requires a much higher dose for administration. The conjugation technique used may be used to create OXM derivatives and other related hormones to obtain long-lasting variants, with improved suitability for clinical use.

**SW04.S16–178**

Identification of novel chemotypes of H<sup>+</sup>, K<sup>+</sup>-ATPase inhibitors

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Proton pump inhibitors (PPIs) are the most prescribable drugs for the treatment of a range of gastrointestinal disorders. The PPIs’ physiological effect is based on irreversible inhibition of H<sup>+</sup>,K<sup>+</sup>-ATPase that leads to gastric acid secretion blocking. Despite of
recognized efficiency different side effects and risks are associated with long-term PPI therapy: inhibition of liver cytochromes [1], morphological side effects [1], relations between PPI use and gastric cancer incidence [2], risk of pneumonia, bone fractures and enteric infections [3], interaction with clopidogrel [3]. Clinically used PPIs are pyridylmethylsulphonyl benzimidazole derivatives that undergo acid catalyzed chemical rearrangement in vivo providing active thiophilic species. These intermediates react with cysteine residues of H⁺,K⁺-ATPase reducing enzymatic activity. Novel chemotypes of H⁺,K⁺-ATPase inhibitors may overcome drawbacks and risks of the currently used agents.

Using ligand-based and structure-based drug design approaches we constructed a small-molecule library of potential H⁺,K⁺-ATPase inhibitors.

Biological testing was performed on membranes enriched for the tubulovesicles of resting parietal cells from rat gastric mucosa. Gastric H⁺,K⁺-ATPase activity was determined by enzymatic method. In primary tests synthesized compounds were utilized at 50 μM concentration in 1% DMSO solution. Three of eighteen tested compounds were active at these conditions. The most active compound, KUD119, was studied on H⁺,K⁺-ATPase inhibition at a concentration-dependent manner. IC₅₀ for KUD119 constitutes 45 ± 0.7 μM at pH 7.2. At 90 μM KUD119 completely suppresses H⁺,K⁺-ATPase activity.

The computational modeling of ligand–protein interactions was undertaken to rationalize inhibitory activity of the newly discovered H⁺,K⁺-ATPase inhibitors. Several favorable interactions of KUD119 with the enzyme active site were determined. The structural data will be integrated in the design of more potent H⁺,K⁺-ATPase inhibitors.

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References

SW04.S16–179
New polymeric nanocarriers for curcumin encapsulation, in vitro release and biocompatibility
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Polymeric nanoparticles have been extensively studied as bioactive carrier agents in the biochemical and biotechnological field. Poorly water-soluble photosensitizing and agents, including naturally occurring curcumin can be used in many encapsulation technologies to improve their several favorable biological properties, such as nontoxicity, dissolution performance, photodynamic activity and biocompatibility by means of template-mediated and self-assembly processes, i.e. subsequent adsorption (layer-by-layer, LbL) of polyelectrolytes and interfacial nanoprecipitation [1–3].

In our study we provided properties of two different types of polymeric nanocarriers, i.e., oil core multilayer nanocapsules prepared via LbL adsorption of sugar-based polyelectrolytes (chitosan and dextran sulfate sodium salt) directly on the nanoemulsion core and poly(D,L-lactide) nanoparticles obtained via nanoprecipitation method, loaded with different types of hydrophobic photosensitizers, i.e., curcumin and the IR-780 cyanine-dye (for comparison) upon their physicochemical characterization, colloidal stability and biocompatibility studies. All experiments were evaluated via photobleaching rate, reactive oxygen species (ROS) generation, in vitro drug release as well as an interaction of loaded and empty nanocarriers with human serum albumin (HSA) and their internalization by macrophages (FACS analysis).

All nanocarriers with different thicknesses of the polymer shell and average size 20–120 nm (studied by DLS, SEM and AFM) demonstrated good capacity for both photosensitizers encapsulation as well as their sustained release in physiological condition (PBS, pH 7.4). Furthermore, our results showed that the photostability and ROS generation by excited curcumin and IR-780 were significantly improved through their encapsulation into polymeric nanoparticles. We also demonstrated that HSA adsorption and cellular uptake of studied delivery system depended upon nanocarrier and loaded photosensitizing agent type.

References

SW04.S16–180
Identification of molecular mechanisms mediating the adjuvanticity of cyclic di-nucleotides
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Vaccination is an important tool in combating infectious diseases. Nevertheless, current vaccination strategies need urgent improvement. Vaccine formulation is based on pathogen subunits and adjuvant molecules that enhance immune response. Vaccines using cyclic di-nucleotides (cyclic di-GMP, cyclic di-IMP and cyclic di-AMP, respectively) as adjuvants have been shown to promote a balanced humoral and cellular immune response. Cyclic di-nucleotides are of low toxicity and are strong mucosal adjuvant candidates. Despite of the fact that their stimulatory effects in mouse immunization experiments have been well described, underlying mechanisms of their activity still remain unclear.

The aim of this project is to clarify molecular mechanisms mediating the adjuvanticity of cyclic di-AMP. For this purpose, in vitro cellular models and immunologically relevant readouts have been established. Primary bone marrow cells and macrophage or dendritic cell line have been identified as responders to c-di-AMP and the regulation of T-cell co-stimulatory molecules, as well as IFN-β production, have been analyzed on those cells. We identified bone marrow derived dendritic cells as major responders to c-di-AMP. Cellular internalization of c-di-AMP has been tested using 2’Fluo-AHC-c-di-AMP. Assays using specific inhibitors have suggested some enzymes to be involved in the activation of antigen presenting cells upon treatment with c-di-AMP.

Insight into investigated mechanisms would facilitate clinical development of cyclic di-nucleotides as adjuvants and prediction of their safety risks. Finally, it may be possible to exploit the resulting molecular targets or developed tools for screening and identification of new compounds exerting immune modulatory activities.
Abstracts

SW04.S16–181
Protective effects of antioxidants against indomethacin-induced tongue injury in rats
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Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the medicines most frequently prescribed in regular practice, and are used for the suppression of fever, pain, and inflammation in various acute and chronic diseases, such as headache and musculoskeletal pain. Reactive oxygen species are well known to play a major role in the etiology and pathophysiology of human diseases. NSAIDs such as indomethacin have pro-oxidant activity and initiate lipid peroxidation by generating reactive oxygen species. Dietary antioxidants are widely used to prevent oxidative stress in the cells. In this study, we aimed to investigate the biochemical effects of antioxidants on indomethacin-induced tongue injury in rats. Male Sprague-Dawley rats were randomly divided into four groups. Group I: intact animals. Group II: control animals receiving vitamin C (vit C) (100 mg/kg), vitamin E (vit E) (100 mg/kg), beta-carotene (15 mg/kg) and sodium selenate (Se) (0.2 mg/kg) for 3 days orally. Group III: animals receiving 25 mg/kg indomethacin. Group IV: animals receiving vit C, vit E, beta-carotene and Se orally for 3 days (in the same dose and time). 2 hours prior to the administration of indomethacin. 6 hours after indomethacin administration all the animals were sacrificed. Tongue tissue samples were taken and homogenized in 0.9% saline to make up to 10% homogenate. The homogenates were used for determining protein, glutathione (GSH), lipid peroxidation (LPO), protein carbonyl levels (PC) and enzyme activities. Tongue GSH levels, superoxide dismutase and glutathione peroxidase activities were decreased, LPO and PC levels, lactate dehydrogenase and myeloperoxidase activities were increased in indomethacin group. Treatment with antioxidants reversed these effects in indomethacin group. The present study showed that vit C, vit E, beta-carotene and Se exerted antioxidant effects and consequently may prevent tongue damage caused by indomethacin.

SW04.S16–182
Selection and properties of 2′-modified RNA aptamers against MBP-specific autoantibodies from patients with multiple sclerosis
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Multiple sclerosis (MS) is a progressive demyelinating autoimmune disorder of central nervous system. It is now established that myelin-specific proteolytic autoantibodies, particularly antibodies against myelin basic protein (MBP) are involved into the development of demyelation and disease progression due to their proteolytic activity [1,2]. Anti-MBP autoantibodies were proposed as biomarkers for diagnosis and clinical prognosis of MS.

The objective of the present work was the in vitro selection of 2′-fluoro modified RNA aptamers for the specific targeting of anti-MBP autoantibodies. To this purpose, polyclonal IgGs were isolated from sera of MS patients, fractionated using MBP affinity chromatography, and used as a target in SELEX procedure. To produce RNA aptamers, we used a SELEX protocol including immunoglobulins’ adsorption to the PCR tube walls and the performance of the whole selection cycle in one PCR tube. After 10 rounds of selection an enriched 2′-F-RNA library with enhanced affinity to target antibodies was cloned and sequenced. A series of individual aptamers was screened for their binding ability. As a result, 71-nt aptamer 2-9 was chosen as a best candidate for the subsequent studies due to high affinity and specificity to target antibodies as compared with IgGs from healthy donors. After sequence minimization, a 3′-shortened variant of 2-9 was obtained. This chemically synthesized 57-nt 2′-F-RNA aptamer 2-9-I demonstrated even better binding affinity than full-length analog 2-9 together with an increased specificity (K_a = 1.2 nM for anti-MBP autoantibodies and over 2000 nM for IgG from healthy donors). Minimized aptamer 2-9-1 was employed then as a basis of fluorescent probes for detection of pathogenic anti-MBP autoantibodies.

To conclude, we obtained 2′-F-RNA aptamers capable of specific recognizing of anti-MBP autoantibodies that could be employed as a basis of new precise tools for MS diagnostics.

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References

SW04.S16–183
Human recombinant polymorphic variants of CYP2C9 and CYP2C19 and its application to pharmacogenetic studies
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Cytochrome P450s are a superfamily of oxygen-activating enzymes, which plays an important role in processes of chemical modification of endogenous and exogenous compounds within human body. P450s, belonging to CYP1, CYP2 and CYP3 families, have a central role and are responsible for the metabolic clearance of up to 75% of all drugs that undergo Phase I metabolism.

CYP2C9 and CYP2C19 are the most important CYPs in the CYP2C subfamily. Together with CYP2D6 they carry out about 40% of the drug metabolism. Those two proteins are highly polymorphic. For CYP2C9 about 40 SNPs have been characterized including the two most common (in Caucasians) variants CYP2C9*2 and CYP2C9*3, and for CYP2C19 – about 30 SNPs, including CYP2C19*2 and CYP2C19*3. Those SNPs caused production of intact enzymes with reduced enzymatic activity arising from amino acid substitutions that are at positions critical for activity.

We have developed a technology for producing human recombinant CYP2C9 and CYP2C19 and their polymorphic variants. We investigated the influence of azole drugs on activity of those CYPs. Azoles, prescribing together with other therapeutically administrated drugs (substrates for those CYPs), can influence the pharmacokinetics and pharmacodynamics of these drugs, leading to the development of a significant number of adverse drug reactions. We found out that some widely used azoles such as miconazole, clotrimazole, ketoconazole, econazole bind tightly to CYP2C9 and its polymorphic forms and decrease metabolism of CYP2C9 substrates very critically. So prescribing azoles together with drugs with narrow therapeutic indices, such as (S)-warfarin, tolbutamide and phenytoin need genotyping the patient and correction does of these drugs. For this purpose we have developed PCR-based methods for routinely genotyping of patients on CYP2C9 and CYP2C19 polymorphisms.
These approaches allow us to determine the contribution of individual polymorphic forms of human CYP2C9 and CYP2C19 enzymes in the metabolism of the most common and newly developed drugs and to choose personalized scheme of pharmacotherapy.

SW04.S16–184
Compounds stabilizers crystals nano calcium carbonate (CaCO₃) preparation with membrane contactor
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The materials made of the same particles, but of smaller size, have different properties from the objects in the micrometer scale. With decreasing size observed changes of chemical, biological, mechanical and optical properties compared to the structures of macro or macro scale [1].

Currently, fine-grained CaCO₃ has found a number of applications (PVC products, paper industry), in which properties change related to size reduction have been used. Moreover, studies at application of nanometric CaCO₃ as an ingredient in pharmaceutical technologies (PVC products, paper industry), in which properties change have different properties from the objects in the micrometer scale. With decreasing size observed changes of chemical, biological, mechanical and optical properties compared to the structures of micro or macro scale [1].

From industrial point of view methods based on the formation of crystals of CaCO₃ in gas-liquid-solid systems are of the highest importance. In this work finecrystalline CaCO₃ was obtained by absorption with chemical reaction [3].

Dimension of particles can be controlled by the introduction of ions into the solution surrounding (Mg²⁺, Cl⁻) or organic macromolecules (alcohols), which block the formation of larger particles. The presence of these compounds allow to control the degree of saturation of the reaction and causes inhibition of crystal growth. Aim of the study was to investigate the potential of stabilizing properties of citric acid. The process was carried out by using a membrane contactor with polypropylene capillary in gas-liquid system. Size of the resulting crystals were analyzed for the two samples of calcium hydroxide concentration ([Ca₄Ca(OH)₂] = 0.076 mM), where in the second of them 0.050 g citric acid was added. In the first case particles CaCO₃ about diameter 1.51 μm was obtained. In the second case noticed that fine-grained CaCO₃ diameter is about 0.68 μm. On this basis, it was noted that the examined substances allowing the stabilization of the particles during the process. By adding a small amount of these components to the observed behavior of the solution in the crystals smaller than in the samples without these additives.

References

SW04.S16–185
Cytoskeleton is implicated in the glutoxim and molixan effect on intracellular calcium concentration in macrophages
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Redox-regulation of cellular processes in normal and pathological conditions is of particular interest. Disulphide-containing drugs glutoxim⁰ (oxidized glutathione disodium salt with Pt nanoaddition, «PHARMA-VAM», Moscow) and molixan⁰ (complex of glutoxim with inosine) are used in clinics as a wide range immunomodulators and hemostimulators. However, the mechanisms of their cellular and molecular effects are still unclear.

Recently we showed for the first time that glutoxim and molixan increase intracellular Ca²⁺ concentration, [Ca²⁺], due to Ca²⁺ mobilization from thapsigargin-sensitive Ca²⁺ stores and subsequent Ca²⁺ influx in rat peritoneal macrophages. Later it was found that tyrosine kinases, tyrosine phosphatases, phosphatidylinositol kinases, Ras proteins, phospholipase C and protein kinase C are involved in the regulatory effect of glutoxim on [Ca²⁺] in macrophages.

Cytoskeleton elements – actin filaments and microtubules are highly redox-sensitive and easily glutathionylated, therefore they may be targets for glutoxim and molixan. Thus, the aim of the present work was to elucidate the possible involvement of actin and tubulin cytoskeleton in the effect of glutoxim and molixan on [Ca²⁺], in rat peritoneal macrophages.

Using Fura-2AM microfluorimetry we have shown for the first time that preincubation of macrophages with actin filament disrupters (cytochalasin D or latrunculin B) or actin stabilizer calcycin A almost completely inhibited [Ca²⁺], increase induced by glutoxim or molixan in macrophages. Morphological data obtained with the use of rhodamine-phalloidin demonstrated that glutoxim and molixan cause the actin filaments reorganization in macrophages: actin clusters appeared in cytosol and cortical layer became wider and loose. Preincubation of the cells with microtubule disrupters (nocodazole or colcemide) or microtubule stabilizer taxol also prevented glutoxim- or molixan – induced [Ca²⁺] increase.

Thus we found that any modification in the actin and tubulin structure in macrophages modulates glutoxim or molixan effect on [Ca²⁺], in macrophages. It may be concluded that actin and tubulin cytoskeleton is an important player in the glutoxim- or molixan-triggered signaling cascade, which leads to [Ca²⁺] increase in macrophages.

SW04.S16–186
Protective effects of lithium: a new look at an old drug with potential
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Sepsis is the systemic response of an organism against microorganisms and toxins. Lithium is a therapeutic agent used for bipo-
The role of infliximab on paracetamol-induced hepatotoxicity in rats

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Paracetamol has a reasonable safety profile when consumed in therapeutic doses. However, it could induce hepatotoxicity and even acute liver failure when taken at an overdose. Infliximab is a tumor necrosis factor-α (TNF-α) inhibitor agent, which has been developed as a therapeutic agent for TNF-α-mediated disease. It acts by binding and neutralizing TNF. The aim of our study was to evaluate the hepatoprotective activity of infliximab on paracetamol-induced hepatotoxicity and to understand the relationship between the TNF-α and paracetamol-induced liver injury. Fifty-six rats were divided into eight groups as each composed of seven rats: (i) intact, (ii) 7 mg/kg infliximab, (iii) 140 mg/kg NAC, (iv) 2 g/kg paracetamol, (v) 5 g/kg paracetamol + 140 mg/kg NAC, (vi) 2 g/kg paracetamol + 3 mg/kg infliximab, (vii) 2 g/kg paracetamol + 5 mg/kg infliximab and (viii) 2 g/kg paracetamol + 7 mg/kg infliximab groups. Liver function tests including lipid peroxidation levels were analyzed and histopathological changes of liver were also observed. There were statistically significant increases in the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), levels of TNF-α and malondialdehyde (MDA) and decreases in the activity of superoxide dismutase (SOD) and level of glutathione (GSH) in the group treated with paracetamol. Infliximab administration dramatically reduced serum ALT, AST and TNF-α level. Also, it restored GSH, SOD and decreased MDA levels in liver. Liver histopathological examination showed that infliximab administration antagonized paracetamol-induced liver pathological damage. The results of present study suggest that infliximab has significant hepatoprotective activity on paracetamol-induced hepatotoxicity.

SW04.S16–188
Plant extracts and some chemical compounds as carbonic anhydrase inhibitors

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Carbonic anhydrases (CAs: EC 4.2.1.1) are zinc enzymes acting as efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate. In humans, CAs are present in a large variety of tissues including gastrointestinal and reproductive tracts, central nervous system, kidneys, lungs, skin, eyes and blood. Several CA enzyme inhibitors sulfonamides and their bioisoesters such as sulfamates, sulfamides are in clinical use. All drugs produced side effects, thus justifying the search for natural CA enzyme inhibitors for safety and economical use. CA inhibitors have applications in the design of various pharmacological agents, such as diuretics, antiglaucoma or anticonvulsants agents.

Plant natural products have been the basis of traditional medicine for thousands of years and continue to actively contribute to contemporary drug discovery. The significance of natural products in drug discovery is most evident in the anticancer and anti-inflammatory therapeutic areas.

In this study, the inhibitory effects of some chemical compounds and water extracts prepared from different plants were investigated on the activity of CA which has an important value in health area.

Plant water extracts were prepared filtered and evaporated to dryness in a rotary evaporator. All the extracts were kept at −20°C and dissolved in water before use. Inhibition of CA was investigated using the esterase assay with 4-nitrophenyl acetate as substrate.

It was determined that all the plant extracts and chemical substances used in our study showed good CA inhibitory effect. The results showed that %5 inhibition values of plant extracts and chemical compounds on the CA were increased with increasing concentration.

It can be suggested that several plant extracts and chemical compounds which are potential sources of CA inhibitors may be appropriate to be used as an additional support to drug treatments such as cancer, osteoporosis, hypertension and eye diseases in the field of health.
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(CaOx) crystals in the kidneys. Many treatment options such as drugs, various herbal preparations, surgical removal of the stones, and extracorporeal shock wave lithotripsy have been applied for this condition. The aim of this study is to assess the effects of the drug amlodipine in an experimentally induced urolithiasis rat model. The effect of 5 mg/kg amlodipine was studied in rats that were first treated with 1% ethylene glycol and 1% ammonium chloride for 21 days to induce urolithiasis. The weight differences and the levels of calcium, magnesium, and phosphate were measured in serum and urine. In addition, urine CaOx level was defined and histopathological analyses were performed on the kidneys. Urolithiasis caused a significant increase in both serum and urine parameters compared with healthy rats. Urolithiasis plus amlodipine administration increased the levels of these same parameters. Urine CaOx level was high in urolithiasis rats and was also increased by urolithiasis plus amlodipine administration. The weight of the rats decreased in the urolithiasis plus amlodipine group when compared with the urolithiasis group. Histopathological examinations revealed extensive intratubular crystal depositions and degenerative tubular structures in the urolithiasis group and the amlodipine treatment group. We showed that amlodipine may increase susceptibility to urolithiasis by raising hyperoxaluria and hypercalciumia. Further studies should be performed to elucidate the urolithiasis activity of amlodipine and to confirm the data.

SW04.S16–190
Eucaryotic-type serine/threonine protein kinases: Potential drug biotargets
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In recent years drugs, targeted serine/threonine kinases (STPKs) develop rapidly. The human STPK Pim-1 is one of the most promising objects studied in this direction [1, 2]. Currently bacterial STPKs also considered as a potential biological targets. It was founded, that many pathogens have STPK playing a key role in their survival. Existing approaches to screen inhibitor libraries of specialized human protein kinase can be used to create antibacterial drugs.

Subjects of our study are STPKs Pim-1 Homo sapiens and PknB Mycobacterium tuberculosis. Our task was to obtain active samples of these enzymes in the E. coli. We have developed the expression, isolation and purification methods for these protein kinases. We have obtained active enzymes that have been used in experiments for screening libraries of small molecules as a potential kinase inhibitors. Enzyme activity was tested at automated workstation Beckmann Biomek 3000, using oligopeptides SGRARTSSFAEPGGK and ITVAELTGVIP as a substrate for Pim-1 and PknB respectively. We were established the ability our protein kinases to autophosphorylation.

We were developed the system without substrate for screening potential inhibitors STPKs at automated station Beckmann Biomek 3000. The system bases on the inhibition of protein kinase autophosphorylation. We use our system for screening libraries of compounds of indolylmaleimides and azolotetrazines classes. The system validated on standard STPK inhibitor mitoxantrone. This system allowed us to select the leader compounds that can be considered as the basis for the creation of potential drugs.

References


SW04.S16–191
WNT pathway activation – new perspective in downregulation of TGF-beta profibrotic action in bronchial asthma
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Bronchial asthma is one of the most common chronic lung diseases. It is inflammatory disorder of the lower airways manifested by reversible constriction of bronchi but also leading to changes in the bronchial wall, sometimes irreversible. Many different factors, including cytokines, growth factors and extracellular matrix proteins are involved in bronchial wall remodeling during the course of asthma. One of the most important signaling molecules is TGF-β that stimulates the process of fibroblasts-to-myofibroblasts transition (FMT).

We used human primary cell cultures, a technique widely used to investigate the pathomechanism of diseases and the search for novel therapy targets. Our previous studies demonstrated that human bronchial fibroblasts (HBFs) derived from patients with diagnosed asthma display predestination towards TGF-β-induced phenotypic switches.

Here we show that activation of WNT pathway by inhibition of GSK-3β activity significantly attenuated TGF-β-induced FMT in HBFs from asthmatics, whilst an opposite effect was observed in HBF isolated from non-asthmatics. The use of GSK-3β kinase specific inhibitor resulted in inhibition of phospho-Smad-2 translocation to the nucleus in asthmatic HBFs and thereby decreased TGF-β ability to exert its function. Moreover, we demonstrated that asthmatic HBFs are capable of connective tissue growth factor (CTGF) synthesis and secretion when stimulated with TGF-β. By activation of WNT signaling pathways using GSK 3β inhibitor, this CTGF expression was diminished.

Our data indicate, that contribution of WNT signaling pathway in the regulation of FMT during asthmatic process is a possible target for therapeutic intervention. The observed cross-talk between WNT and TGF-β signaling pathways in HBFs isolated from patients with bronchial asthma modulates auto- and paracrine production of CTGF, a cytokine critically involved in fibrotic diseases, which is the novelty of our findings.

SW04.S16–192
NO-releasing xanthine KMUP-1 bonded by simvastatin attenuates bleomycin-induced lung inflammation and delayed fibrosis
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Background and purpose: Pulmonary fibrosis (PF) is a progressing lung injury initiated by pulmonary inflammation (PI). Bleomycin (BLM) is the most common pathogenesis of PF through early PI and extensive extracellular matrix deposition. This study is aimed to determine whether NO-releasing KMUP-1 inhibits PI and PF, and if so, the benefits of KMUP-1S resulted from simvastatin (SIM)-bonding to KMUP-1.
Experiment approach: C57BL/6 male mice were intra-tracheally administered BLM (4 U/kg) at day 0. KMUP-1 (1–5 mg/kg), KMUP-1S (2.5 mg/kg), SIM (5 mg/kg). Plus (KMUP-1 2.5 mg/kg + SIM 2.5 mg/kg) and clarithromycin (CAM, 10 mg/kg) were orally and daily administered for 7 and 28 days, respectively, to mice, sacrificed at day-7 and day-28 to isolate the lung tissues, for examining the inflammatory and fibrotic signaling and measuring the cell population and MMP-2/MMP-9 activity in bronchovascular lavage fluid (BAL).

Key results: KMUP-1 and KUP-1S significantly decreased neutrophil counts in BAL fluid. Fibroblastic foci were histologically assessed by H&E and Masson’s trichrome stain and treated with KMUP-1 and references. Lung tissues were determined the contents of collagen and the expressions of TGF-β, α-SMA, HMGB1, CTGF, eNOS, p-eNOS, RhoA, Smad3, p-Smad3, MMP-2 and MMP-9 by Western blotting analyses, respectively. These changes are regulated by NO/cGMP and inhibited by various treatments. KMUP-1 and KMUP-1S predominantly prevented HMGB1/MMP-2 expression at day-7 and reduced TGF-β/p-Smad3 phosphorylated Smad3 and CTGF at day-28.

Conclusions and implications: KMUP-1 and KMUP-S restore normal lung tissue structure and reduce BALF inflammatory cells, potentially useful for the treatment of BLM-lung PF.

SW04.S16–193
In vitro biocompatibility studies of cyanine-loaded poly(D,L-lactide) nanocarriers; hemolytic activity, macrophage uptake and interaction with serum albumin
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Successful drug targeting using nanoparticle delivery system can be achieved solely if the nanoparticle remains in the blood stream long time enough to accumulate at its site of action, for instance a tumorous or inflamed tissue. Parenteral administration of drug nanotransporters induces body biological response where many factors may influence biodistribution of delivered drug. The nanoparticles in the blood stream immediately encounter a complex environment of plasma proteins and immune cells and also may interact with red blood cells [1]. In this report we described the physicochemical properties and biocompatibility evaluation of poly(D,L-lactide) (PLA) nanospheres stabilized by Cremophor EL and loaded with the hydrophobic cyanine-type photosensitizer IR-780 and ZnPc, obtained by nanoprecipitation method [2]. DLS measurements confirmed the particle diameter below 150 nm and AFM and SEM – its morphology and shape. The Doppler electrophoresis measurements provided a negative ζ-potential of obtained nanoparticles. UV-Vis spectrophotometry was applied to determine the encapsulation efficiency (about 90%). In vitro dye release profiles showed long-term colloidal stability of the obtained nanospheres. Hemolytic potential of Cremophor EL/PLA/water nanospheres, determined in whole human blood by a standard colorimetric method [3], does not exceed value of 2% indicating hemocompatibility of the investigated nanocarriers. The nanospheres uptake by macrophage cells was observed by flow cytometry based on fluorescence emission of excited cyanine IR-780 or ZnPc. The internalization yield of the ZnPc-loaded nanospheres was about fivefold lower than that containing IR-780 which observation proves that such nanocarriers would have potentially longer half-life in the circulation. The plasma proteins adsorption at the nanoparticle surface may be of a critical character in relation to phagocytic recognition by immune system cells. Because serum albumin is the most abundant protein in blood plasma, we determined its influence upon the nanosphere internalization in macrophages. The albumin presence decreased in ca. 22–26% the nanospheres cell uptake. The same differences in the protein absorption ability at the nanocarriers surface was observed after immobilization of albumin on a 96-well plate. Furthermore, formation of complexes of serum albumin with nanospheres was confirmed by 1HNMRR techniques.

References

SW04.S16–194
Differential changes in protease-antiprotease balance and serum levels of soluble tumor necrosis factor receptors during radioiodine therapy
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Background: In papillary thyroid carcinoma (PTC) with/without autoimmune thyroid diseases (AITD), the balance between expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) is broken. After radioiodine (I-131) therapy, tumor necrosis factor-alpha (TNF-α) precursor is cleaved to release soluble TNF-α to increase apoptosis. TNF-α can be slowly cleaved from the cell surface by MMPs, producing a bioactive cytokine, soluble TNF-α, which may increase apoptosis through binding to the TNF receptor type I (TNFRI). We aimed to evaluate the effects of therapeutic irradiation with I-131 on serum levels of MMP-9, TIMP-1, TNF-α, soluble TNFRII (sTNFRII) and TNFRI (sTNFRI), in PTC and PTC associated with AITD (PTC+AITD) patients.

Methods: We selected 54 patients with PTC (8M/46F) and 41 with PTC+AITD (3M/38F). PTC+AITD patients had positive titers of anti-Tg autoantibodies (TgAb). Peripheric blood samples were collected before and at 96 hours after I-131 administration. The serum levels of TgAb, TNF-α, sTNFRI, sTNFRII, MMP-9 and TIMP-1 were measured by ELISA.

Results: I-131 therapy of PTC+AITD patients was associated with an increase with 18% in TgAb level (p = 0.001), 5% in MMP-9/TIMP-1 ratio (p = 0.003) and a decrease with 29% in TNF-α/sTNFRI ratio and 31% in TNF-α/sTNFRII ratio (p < 0.001). TgAb titters are positively related to MMP-9/TIMP-1 ratio (r = 0.52, p < 0.01). In PTC group, the beneficial effect of I-131 was illustrated by a significant reduction of MMP-9/TIMP-1 ratio with 44% (p = 0.003) and an increase with 66% in TNF-α/ sTNFRII ratio and 61% in TNF-α/sTNFRI ratio (p < 0.001). The reduction of MMP-9/TIMP-1 ratio is positively correlated with sTNFRI level at follow-up (r = 0.67, p = 0.009).

Conclusions: Elevated TNF-α/sTNFRII, TNF-α/sTNFRII ratios indicate a decline in disease activity after I-131 therapy more pronounced in PTC than in PTC+AITD, suggesting that suppression
of sTNFR1, sTNFRII or increased production of TNF-α is required to initiate remission of cancer. In PTC patients, I-131 therapy has almost halved the imbalance between MMP-9 and TIMP-1 and this decrease may reduce tumor cell viability and migratory potential. In PTC+AITD patients, increased TgAb titers partially block the beneficial effect of I-131.

SW04.S16–195
Thermal stability of recombinant human carbonic anhydrases II and VI
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Carbonic anhydrases (CA) are zinc-containing enzymes which catalyze the reversible hydration of carbon dioxide in the reaction $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$. They are essential in all kingdoms of life and humans have 12 active CA isoforms. CA II is a ubiquitous cytosolic enzyme that has relatively high specific activity. CA VI is expressed in salivary glands and secreted to saliva and milk. Decrease of salivary CA VI concentration is involved in dental caries, acid injury of the gastrointestinal mucosa, decreased sense of taste and smell. Diseases caused by CA expression disorders showed that CAs are potential targets for diagnosis and treatment.

Human recombinant CA II (full length) and VI (catalytic domain, 21–280 a.a.) were expressed in E. coli and purified by affinity chromatography. Thermal stability of the enzymes was determined by the thermal shift assay (TSA). TSA is a rapid screening method based on fluorescence change observed upon protein unfolding at elevated temperatures and requires relatively low amount of purified protein. The protein melting temperature, a midpoint of the thermal unfolding transition, depends on the composition of the solution. Various buffers, salts, osmolytes, excipients, and ligands affect the melting temperature by binding to the protein. Characterization of protein thermal stability helps the determination of protein purification and storage conditions. Here we present the pH stability profiles of CAs II and VI in the presence of several buffers and rare metal salts. Citrate was found to significantly destabilize the CA relative to other buffers. Stability profiles are used for potential inhibitor screening and the design of drug-leed compounds.

SW04.S16–196
Staphylococcus aureus-induced sepsis and Coenzyme Q10 therapy: an isolated rat heart study
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Sepsis is defined as a condition in which microorganisms and their toxins are introduced into the bloodstream, and inflammation and the host response coexist. Staphylococcus aureus (S. aureus), a gram positive bacterium, is one of the causative microorganisms in sepsis cases seen in the population. Coenzyme Q10 (CoQ10) plays an important role in oxidative phosphorylation, and protects mitochondrial proteins and DNA against oxidative damage by inhibiting lipid peroxidation in biological membranes.

This study aimed to achieve sepsis in rats by injection of S. aureus, to reveal myocardial dysfunction, and to establish whether CoQ10 plays a protective role against a possible myocardial injury.

In this study, 3-month-old 24 male Wistar albino rats were used. Animals were divided into four groups, septic (15 × 10⁸ CFU/ml S. aureus ip a single dose 24 hours prior to operation), CoQ10 administrated (200 mg/kg/day per os for 7 days until the experiment day) and CoQ10 administrated septic groups. Blood samples were collected from abdominal vein for biochemical and microbiological analysis. Afterwords hearts were attached to Langendorff isolated heart system. Cardiodynamic parameters were recorded during the experiment and also, perfusate samples were collected at defined time points. At the end of the experiment hearts were homogenized for biochemical and microbiological analysis. All data were statistically evaluated.

A considerable amount of bacteria were present in blood and heart tissue samples taken from septic groups. In the group subjected to S. aureus, levels of C-Reactive Protein, myeloperoxidase, N-Terminal-pro-Browniet Peptid, cardiac troponin T and creatine kinase-MB were higher, while total antioxidant capacity was lower. In comparison of cardiodynamic parameters of septic group with the control, perfusion pressure and end diastolic pressure were lower, whereas heart rate, Max dP/dt, contractility index and rate pressure product levels were higher. On the other hand, CoQ10 application positively affected cardiodynamic parameters, in part.

As a result, we suggest that S. aureus-induced sepsis causes myocardial dysfunctions and CoQ10, improved myocardial functions in both control and septic individuals in a dose- and time-dependent manner, partially.

SW04.S16–197
The effects of vitamin B6 on testis injury induced by valproic acid
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Valproic acid (2-propyl-pentanoic acid; VPA) is one of the most frequently prescribed antiepileptic drug worldwide. The drug has multiple modes of action and affects neurologic, endocrine and reproductive system in different ways. VPA is known to have severe toxic effects on testis both in experimental animals and in humans. Vitamin B6 is a water soluble, chemically quite distinct compound. Vitamin B6 plays a primary role acting as a cofactor for a large number of essential enzymes. The aim of this study was investigated the effects of vitamin B6 on VPA-induced testis injury. In this study, male Sprague Dawley rats were used. Rats were randomly divided into four groups. Group I; control animals. Group II; control animals given vitamin B6 (50 mg/kg/day) for 7 days. Group III; animals given VPA (500 mg/kg/day) for 7 days. Group IV; animals given VPA+vitamin B6 (in the same dose and time). Vitamin B6 was given by gavage and VPA was given intraperitonally to rats. On the 8th day of experiment, all of the animals were fasted overnight and then sacrificed under anesthesia. Testis tissue was evaluated using biochemical, immunohistochemical and histological methods. Histological sections were stained with hematoxylin and eosin and caspase-3 activity was carried out immunohistochemically in order to show apoptotic activity. To biochemical examination, testis tissues were homogenized in 0.9% saline to make up to 10% homogenate. The homogenates were used for protein and protein carbonyl contents, and enzyme activities. The germ cells were sloughed as a group into the lumina of most seminiferous tubules in VPA
Efficient, non-toxic gene delivery by negatively charged polypropyl-based lipoplexes: application in RNA delivery and the effects on cell physiology

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The development in the field of DNA and RNA delivery into cells and progress in understanding pathogenesis of many diseases resulted in nucleic acids becoming actually drugs and their delivery one of the top molecular biology techniques applicable in clinics. Still, one of the major challenges facing the development of gene therapy is lack of efficient and safe gene vectors.

We have examined a new class of polypropyl-based cationic lipids for gene transfer. Studies have shown that semisynthetic polypropyltrimethylammonium iodides (PTAI) in formulations with co-lipids (DOPE, DC-cholesterol, DOPC) have the ability to effectively transfet plasmid DNA in a wide range of cell types in vitro both in the presence and absence of serum. Although generally it is considered that bigger lipoplexes bearing positive zeta potential are more efficient, our data clearly demonstrate that small (90–150 nm), negatively charged (about –30 mV) polypropyl-based lipoplexes are efficient and have parameters making them promising candidates for in vivo gene delivery.

As it was demonstrated that lipofection procedure may have several side effects on cell physiology, we tested the effects of PTAI formulation on cell motility, proliferation, viability and gap junctional intercellular coupling (GJIC). We have tested four derivatives: amino-Pren-7, amino-Pren-8, amino-Pren-11 and amino-Pren-15. Cell motility of a model DU-145 (human prostate cancer) cells was estimated by time-laps monitoring of movement of individual cells and GJIC intensity measured using donor cells labelled with calcine plated onto monolayers of acceptor cells transfected with PTAI-based lipoplexes. The dynamics of calcine transfer from donor to acceptor cells was analyzed. Antimicrobial activity was evaluated by colony reuction assay and the hemolytic activity against human red blood cells (RBCs) was tested using PBS suspension prepared from fresh blood.

The results show that lipoplexes based on PTAI have no effects on cell physiology that is cell viability, proliferation and morphology. Moreover, they also occurred to have no effect on GJIC and cell motility (24 hours after transfection all the cells cover the distance of about 210–240 µm showing a displacement of 70–80 µm). Some PTAI-based vectors exhibit potent bactericidal activity against Streptococcus aureus and Escherichia coli, while showing no toxic effect on eukaryotic cells, which can be beneficial during prolonged storage of formulations. Furthermore, (as we suggest in vivo application of PTAI vectors) we have also proved their safety towards human RBBS, which membranes are not disrupted in the presence of all the examined concentrations of PTAI-based lipoplexes. Moreover, the formulations tested in plasmid DNA transfer into cells are also effective in gene silencing techniques utilizing RNA delivery. We have successfully introduced shRNA inducing GFP gene silencing into DU145, XC (rat sarcoma) and B16F10 (mouse melanoma) cells expressing pEGFP-C1 plasmid achieving GFP gene silencing. Additionally, PTAI-based formulations can be safely stored for extended periods (up to 18 months) at 4°C.

In conclusion, lipoplexes based on PTAI provide ability to introduce DNA or RNA into cells with satisfying efficiency, easily and safety, as they exhibit no toxic activity and no side effects on cell proliferation, motility and GJIC. What is more, PTAI-based formulations show advantages important for convenient use (both – DNA and RNA delivery, antimicrobial activity, prolonged storage) and in vivo applications (no RBCs rupture in the presence of PTAI-based lipoplexes, effectiveness in the presence of serum).

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Dermal restructuring effect of Trifolium pratense extract demonstrated by in vitro comparative studies

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The clover extract, named in our studies Dermo ET, comprises as active principles daidzein, genistein, formononetin and biochanin A, thus for that its activity was demonstrated by comparatively evaluating these components’ action together with their combination in the corresponding doses existing in the original extract. The effect was emphasized on standardized cell line of human dermal fibroblasts, Hs27, the main cell type responsible for the structural proteins synthesis from extracellular matrix, the most important being collagen type I and III. There were obtained complementary data for the following mechanisms: collagen synthesis and the rate of cell multiplication’ stimulation, as well as the induced overexpression of α1β1 and α2β1 integrins.

Extracellular matrix remodeling involves a balance between synthesis and degradation of structural components under catalytic action of MMPs. These mechanisms were highlighted by two analysis techniques: determination of the amount of collagen by hydroxyproline dosage and identification of MMP 2 and 9 by gelatin zymography from the cellular samples of Hs27 fibroblasts treated with Dermo ET/phytohormons. There were obtained positive correlative effects with Dermo ET and daidzein.

Stimulation of cell multiplication rate was tested by two complementary techniques of analysis: quantification of cell cycle and succeeding proliferative generations, finding its acceleration induced by the clover extract, Dermo ET (over 30% of the corresponding witness) and also by the equivalent phytohormone mixture.

The balance of expression of integrin α1β1 and α2β1 integrin is important for the degradation/synthesis of collagen and was revealed by flow cytometry using monoclonal antibodies to α and β chains. Tests revealed Dermo ET action, in a dose-effect manner, only on the induction of glycoprotein α2 chain overexpres-
sion, indicating an increase in fibroblast – collagen type I ties and in stimulating collagenase activity in fibrogenesis.

These comparative studies conducted in vitro have shown the positive impact on dermal restructuring of the clover extract and the phytohormons featured in its composition on the human dermal fibroblasts, the major cell type involved in maintaining normal extracellular matrix status.

SW04.S16-200
The regulation of human gamma-glutamyltransferase gene expression
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Gamma-glutamyltransferase (GGT) catalyses the hydrolisis of the gamma-glutamyl bond in glutathione and glutathione S-conjugates. GGT substrates include glutathione, glutamine, S-nitrosoglutathione, leukotriene C4, certain drugs and toxins and therefore are critical in many processes including antioxidant defence mechanisms, inflammation, drug metabolism and detoxification. Serum GGT is routinely used as a biomarker of tissue damage and was shown to be associated with all-cause mortality risk, cardiovascular disease, asthma, metabolic syndrome, Parkinson’s disease, type 2 diabetes and cancer. Despite the increasing number of the research on the subject, the mechanisms behind the regulation of GGT plasma level are still poorly understood. Here, we provide analysis of the transcription factor binding sites of human GGT1 gene and propose signalling pathways involved it the regulation of its expression.

SW04.S16-201
The effect of edaravone on skin antioxidant, oxidant parameters in valproic acid induced toxicity
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Valproic acid (VPA) is an antiepileptic drug and has some adverse effects on gastrointestinal, neurological, hematological and reproductive systems. However, there is no data related to its effects on skin oxidant or antioxidant metabolism. As edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one) is a potent free radical scavenger, the effect of edaravone on skin antioxidant and oxidant parameters in VPA induced toxicity was investigated in the present study. For this purpose, female rats were randomly divided into four groups. Group I was intact control animals. Group II was given only VPA (0.5 g/kg/day, i.p.) for 7 days. Group III was given only edaravone (30 mg/kg/day, i.p.) for 7 days. Group IV was given VPA+ edaravone (same dose and time i.p.). On the 8th day of experiment, all of the animals were fasted overnight and then sacrificed under ether anesthesia. Skin tissues were taken from animals and homogenized. Total protein and glutathione, lipid peroxidation levels and superoxide dismutase activity were determined in tissue homogenates. The results were evaluated statistically and discussed.

SW04.S16-202
The effects of tempol on liver in LPS-induced acute endotoxemia in the rat
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Endotoxemia gives rise to the events including inflammatory and anti-inflammatory process, humoral and cellular reactions and circulatory abnormalities and also, multiple organ dysfunction syndrome because of an increased formation of reactive oxygen species (ROS) The excessive production of ROS in endotoxemia leads to oxidative stress. Interventions, which reduce the production or the effect of ROS, have been shown beneficial effects in a variety of experimental models. These therapeutic strategies include antioxidant enzymes such as superoxide dismutase (SOD) and catalase and radical scavengers. Tempol is a membrane-permeable and radical scavenger that interferes with the formation or the effects of many radicals including superoxide anions, hydroxyl radicals, and peroxynitrate. In this study, we aimed to evaluate the effects of Tempol on liver in endotoxemic rats given lipopolysaccharide (LPS), endotoxin of Gram-negative bacteria. Male Wistar-albino rats were randomized into four groups: Control group: Physiological saline injected; LPS group: given 15 mg/kg LPS (E. coli, 026:B6); LPS+Tempol: 100 mg/kg Tempol administration followed by 3 hours of LPS treatment; Tempol group: 100 mg/kg Tempol injected 3 hours after the administration of physiological saline. Blood was collected by cardiac puncture and liver samples were taken for immunohistochemical and biochemical analysis. Myleperoxidase (MPO)-stained leukocytes were observed in sinusoids, vessel and portal areas in LPS group. iNOS reaction was also observed in hepatocytes, leucocytes and Kupffer cells. iNOS reaction decreased in hepatocytes in Tempol injected group. However, distribution of MPO-stained leukocytes and iNOS reaction of leucocytes did not change. Tempol administration reduced increased levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in LPS group compared with control group. SOD level increased with Tempol administration in LPS group. In conclusion, these results showed that Tempol was effective partially on prevention of oxidative stress.

SW04.S16-203
The protective effect of amiodarone in lung tissue of cecal ligation and puncture-induced septic rats: a perspective from inflammatory cytokine release and oxidative stress
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Sepsis is a serious medical condition that is characterized by a whole-body inflammatory state and the presence of a known or suspected infection. Amiodarone is a class III antiarrhythmic agent, a multichannel blocker (Ca++, Na+, and K+), and a non-competitive α- and β-adrenergic blocker in cardiac cells. The
present study aimed to determine whether amiodarone was protective against experimentally induced cecal ligation and puncture sepsis in rat lung tissue. The relationship between its probable protective effect and antioxidant/anticytokine action biochemically. Five groups of rats were used, each composed of 20 rats: (i) the sham-operated control group; (ii) the CLP group; (iii) the 25-mg/kg amiodarone-treated control healthy group; (iv) the 50-mg/kg amiodarone-treated CLP group; and (v) the 50-mg/kg amiodarone-treated CLP group. A CLP polymicrobial sepsis model was applied to the rats. All groups were sacrificed 16 hours later, and lung and blood samples were analyzed biochemically. Twenty-five and 50 mg/kg amiodarone decreased the level of tumor necrosis factor-α in serum. They increased the activities of superoxide dismutase and levels of total glutathione in lung tissues of rats. Our results indicate that administration of amiodarone prevented oxidative stress and cytokine activity and protected lung tissue during sepsis cascade.

SW04.S16–204
Synthetic muramyl peptides differ in their activity to stimulate the production of iNOS synthase and nitric oxide
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Muramyl peptides are known to be minimal blocks of Gram-positive and Gram-negative bacterial peptidoglycans and realize their immunomodulatory properties via Nod1 and Nod2 intracellular receptors. Numerous studies have defined the role of MPs in host defense, adjuvanticity, inflammation and biochemical processes. Many synthetic analogues of muramyl peptides have been obtained and some of them are registered as medicaments.

Here, we analyzed the amount of released nitric oxide and inducible NO synthase after stimulation of macrophage cell line RAW264.7 by LPS and eight synthetic glucosaminylmuramyl peptides (GMDPs) analogs that had been screened previously for their adjuvanticity and cytokine activity. Using a Griess Reagent System and RT-PCR analysis, we demonstrate on the macrophage cell line RAW264.7 that modification of the second amino acid of GMDP generates muramyl peptide derivatives with enhanced NO-activating capacity while modification of the saccharide part of GMDP does not influence on it. Some of these GMDPs have prolonged action.

These findings offer a basis for the development of new drugs that could be used in the treatment of inflammatory disorders that have been associated with Nod2 dysfunction and for chronic stimulation of Nod2 in mediating tolerance to bacterial products.

References

SW04.S16–205
Biological relevance of fluorescent trilobolide conjugates
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The relevance of fluorescently labeled natural products for molecular biology and medicine has been increasing since variety of information has become available: the cellular environment of the molecule, its orientation and/or association with other molecules. Particular progress has opened new possibilities for tracking the activity of natural products in animal tissues.

Trilobolide (Tb), structurally related to thapsigargin, is a sesquiterpene lactone isolated from Laser trilobum (L.) Borkh. From medicinal point of view Tb is a potent immunostimulatory agent. Tb is a strong inducer of IFN-γ secretion and it is associated with NO production in PBMC cells. Also, the dependence of its stimulatory potential on activation of MAP kinases p38, ERK 1/2 and transcription factor NF-κB has been described as well as its inhibitory activity to sarco/endoplasmic Ca2+ ATPase (SERCA).

We synthesized and studied fluorescently labeled trilobolide with green-emitting BODIPY dye attached by spacers of different lengths. The live-cell-imaging experiments of the prepared bioconjugates brought clear evidence of their localization in endoplasmic reticulum of prostate cancer cell-lines. Metabolic activity and NO production of cell treated with Tb derivatives were also tested.

SW04.S16–206
Cellular calcium dyshomeostasis and neurodegeneration
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The signalling function of calcium is particularly important to neurons. They control the ion by transporting it across membranes, by regulating its entrance into the cytoplasm through various channel types, and by complexing it to soluble proteins that also have the function of decoding its message (calcium sensors).

The calcium signal, however, is ambivalent: it becomes a conveyor of doom if its movements and concentration within cells are not carefully controlled. Massive dysregulations of cell calcium are incompatible with cell life and rapidly end with cell death. Subler dysfunctions of one or more of the calcium controlling systems disturb normal neuronal cell life generating phenotypes with different degrees of severity. Calcium dyshomeostasis is increasingly considered to be at the basis of neurological diseases of great importance, e.g., Huntington’s and Alzheimer’s diseases, and of a number of channelopathies and of ataxias. Our Laboratory has concentrated on molecular neuronal defects in Huntington’s disease (HD) and in cerebellar ataxias. On HD we have discovered defects in the handling of calcium by mitochondria in a number of neuronal types. We have also found severe changes in mitochondrial dynamics in HD model neurons and in lymphoblasts of HD patients: mitochondria are increasingly fragmented and exhibit alterations of the cristae. The increased cytosolic calcium activates calcineurin which dephosphorylates the pro-fission protein Drp1, favoring its translocation to mitochondria. The alterations of mitochondrial dynamics increases the apoptosis propensity of the neurons.

We have recently discovered an X-linked form of cerebellar ataxia caused by a mutation in isof orm 3 of the plasma membrane calcium pumps (PMCA). The mutation (a G/D replacement)
SW04.S16–207
Pharmacogenomics of multiple sclerosis
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Immunomodulatory drugs are currently used for long-term medication of multiple sclerosis (MS). Interferon-beta (IFNb) and glatiramer acetate (GA) are the first-line disease modifying treatments (DMTs), which have been shown to improve MS course. However, a significant share of MS patients experiences modest benefit from DMT therapy. Pharmacogenomic studies in MS have been actively focused on IFNb and GA therapy in order to discover predictive genetic biomarkers of MS treatment efficacy. However, the modest association of allelic polymorphism of distinct candidate genes with treatment efficacy has been found, which may result from the polygenic background of MS treatment response. These studies should be extended in enlarged groups of MS patients, selected in compliance with their ethnic homogeneity.

We have performed pharmacogenomic studies of IFNb and GA treatment efficacy in Russian MS patients. The whole-genome transcriptome analysis (Illumina HT-12) has predictably shown the differential expression of immune-response genes before and after treatment and elucidated the most promising genes for the following analysis. Pharmacogenetic study based on identical clinical criteria of FINb and GA treatment response was performed for the polymorphic loci of nine immune-response genes in 508 patients. Taking into account the small effect of individual genetic contribution, we investigated joint contribution of multiple allelic variants applying APSampler algorithm. Some highly-specific composite markers of IFNb response and GA response were identified. Discriminative composite genetic markers for choosing either of two DMTs were found in a comparative pharmacogenetic analysis.

Based on these findings we have also scheduled the approach which allows interpreting the character of cumulative effect(s) of allelic variants in the found composite markers of DMT response. It was shown that observed cumulative effects were the result of both additive effects and epistatic interactions between allelic variants. Discovered composite genetic markers provide a possibility for selection of preferable first-line DMT for individual MS patients before treatment.

SW04.S16–208
S250F variant associated with aromatic amino acid decarboxylase deficiency: molecular defects and intracellular rescue by pyridoxine
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Dopa or aromatic amino acid decarboxylase (DDC, AADC) is a pyridoxal 5'-phosphate-dependent enzyme that catalyzes the production of the neurotransmitters dopamine and serotonin. Among the so far identified mutations associated with AADC deficiency, an inherited rare neurometabolic disease, the S250F mutation is the most frequent [1–4]. Here, for the first time, the molecular basis for the functional deficit of the S250F variant was investigated both ‘in vitro’ and in cellular systems. Although Ser at position 250 is not essential for the catalytic activity of the enzyme, its mutation to Phe causes a ~7-fold reduction of catalytic efficiency and a conformational change in the proximity of the mutated residue transmitted to the active site. In cellular extracts of ’E. coli’ and mammalian cells, both the specific activity and the protein level of the variant decrease with respect to the wild-type. The results with mammalian cells indicate that the mutation does not affect intracellular mRNA levels and are consistent with a model where S250F undergoes a degradation process via the proteasome, possibly by the ubiquitin system, occurring faster than the wild-type. Overall, biochemical and cell biology experiments show that loss of function of S250F occurs by two distinct but not exclusive mechanisms affecting activity and folding. Importantly, exposure of mutant-expressing cells to 4-phenylbutirric acid (4-PBA) or, to a major extent, pyridoxine increases the expression level and, in a dose-dependent manner, the specific activity. This strongly suggests that 4-PBA and/or pyridoxine administration may be of important value in therapy of patients bearing S250F mutation.

References

SW04.S16–209
Association of MAOA, CCK, COMT, TPH1, SERT, PDE4B with panic disorder in patients from the Moscow population
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Background: Panic disorder (PD) is a disease characterized by the appearance of spontaneous panic attacks a few times a year, up to several times per day, and fear out of their occurrence. Genetic factors play a role in the pathogenesis of PD (heritability value 48%). However, the molecular-genetic basis of the disease have been studied very little. Published data indicate that polymorphic variants of genes encoding key enzymes of neuromediatory systems, neurotransmitters and their receptors play a role in development of PD. We have selected the basic proteins, gene polymorphisms that may have an impact on the development of PD:
neuropeptide cholecystokinin (CCK); monoamine oxidase A (MAOA); catechol-O-methyltransferase (COMT); solute carrier family 6 (SLC6A4 or 5-HTT); serotonin transporter (SERT), tryptophan hydroxylase 1 (TPH1); phosphodiesterase 4B (PDE4B).

**Objectives:** Assess the contribution to the development of PD the alleles: STR in 5′-region of CCK gene, VNTR in 5′-region of MAOA gene, SNPs in genes COMT (rs4680), 5-HTT (rs3813034), SERT (rs3813034), TPH1 (rs1800532), PDE4B (rs502958) in rs1040716).

**Methods:** Blood samples of patients diagnosed with PD (n = 82) and blood samples of unexamined people (control) living in Moscow and the Moscow region (n = 192). STR and VNTR alleles distinguished by fragment analysis, SNPs detected by Real-Time.

**Results:** An analysis of STR in CCK gene and the VNTR in MAOA gene was identified combination of alleles (S/M) is more common in a sample of patients with PD compared with control sample. Genotype AC of SNP rs3813034 (SERT) – is less common in patients with PD, compared with the control samples. Allele frequencies of gene COMT, 5-HTT, TPH1 and PDE4B did not differ between the groups of patients and controls.

**Conclusions:** These data suggest that polymorphisms in CCK, MAOA and SERT genes can be associated with PD. Further studies could highlight the association between these polymorphisms and the appearance of panic disorder.

**SW04.S16–210**

Curcumin inhibits transthyretin extracellular deposition *in vivo*: implications for Familial Amyloidotic Polyneuropathy (FAP) therapy

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The transthyretin amyloidoses (ATTR) are devastating diseases characterized by progressive neuropathy and/or cardiomyopathy for which novel therapeutic strategies are needed. We have recently shown that curcumin ( diferuloylmethane), the major bioactive polyphenol of turmeric, strongly suppresses TTR fibril formation in vitro, either by stabilization of TTR tetramer or by generating nonfibrillar small intermediates that are innocuous to cultured neuronal cells [1].

In the present study, we aim to assess the effect of curcumin on TTR amyloidogenesis in vivo, using a well characterized mouse model for Familial Amyloidotic Polyneuropathy (FAP). Mice were given 2% (w/w) dietary curcumin or control diet for a 6 weeks period. Curcumin supplementation resulted in micromolar steady-state levels in plasma as determined by LC/MS/MS. We show that curcumin binds selectively to the TTR thyrsmic-binding sites of the tetramer over all the other plasma proteins. The effect on plasma TTR stability was determined by isoelectric focusing (IEF) and curcumin was found to significantly increase TTR tetramer resistance to dissociation. Most importantly, immunohistochemistry (IHC) analysis of mice tissues demonstrated that curcumin reduced TTR load in as much as 70% and lowered cytotoxicity associated with TTR aggregation by decreasing activation of death receptor Fas/CD95, endoplasmic reticulum (ER) chaperone BiP and 3-nitrotyrosine in tissues.

Taken together, our results highlight the potential use of curcumin as a lead molecule for the prevention and treatment of TTR amyloidosis.

**Reference**


**SW04.S16–211**

Antibodies to acetylcholine receptor and prion protein protect cells from amyloid-beta induced toxicity and preserve memory impairment in mouse model of Alzheimer

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Alzheimer’s disease (AD) is characterized by many pathological features such as memory impairment, neuronal loss and amyloid plaque formation in cortex and hippocampal area of the brain [1]. It was found that amyloid-beta (Abeta) is responsible for such intracellular dysfunctions as reactive oxygen species (ROS) production, increasing in Ca²⁺ -signaling and caspase 3 activation [2,3]. Abeta was shown to mediate all these processes via binding with several proteins on the surface of the brain cells. Alpha7-type of the acetylcholine receptor (AChR) and the prion protein are shown to be involved in pathogenic binding with Abeta [4,5]. We suggested that the antibodies specific to these proteins can be a prominent tool for development of an anti-AD treatment. We showed that induction of specific antibodies against peptide 173–193 of alpha7-subunit AChR or against fragment 95–123 of the prion protein leads to memory improvement in mice with the experimentally induced form of AD, prevents plaque formation in the brain of the mice and improve morpho-functional state of neurons in cortex. Injection of affinity purified antibodies to the found fragments also had the positive effect of on the memory of the experimental mice. The activity of the antibodies against both fragments was investigated on cortical or hippocampal co-cultures of neurons and astrocytes treated with Abeta. It was demonstrated that preincubation of the co-culture with both antibodies decreased the number of dead cells after treating with Abeta. It was also shown that the observed effect was Ca²⁺ -independent – the presence of both types of affinity purified antibodies did not block the influx of Ca²⁺ into the cells. However, both antibodies decreased the rate of ROS production in cells treated with Abeta. This effect was accommodated by decreasing in the rate of NADPH-oxidase activity and was associated with lowering in caspase 3 activation. The results obtained prove the idea that induction of antibodies against Abeta receptors is a perspective approach to AD therapy. To prove that fact in our further studies we investigated an effect of antibodies against RAGE protein which is also known to moderate Abeta toxic effect on brain cells.


**References**

From evolution to pharmacology: developing agonists of neurotrophin receptors

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Neurotrophins control brain development and maintenance during adulthood and in aging. They act through prosurvival tyrosine kinase Trk and pan-neurotrophin p75NTR receptors, exerting potent neuroprotective and neurogenic effects in various neurodegenerative diseases. Unfortunately, their polypeptidic nature limits their therapeutic potential. We have recently shown that neurosteroid dehydroepiandrosterone (DHEA), prevents neuronal apoptosis (Charalampopoulos et al., PNAS 2004), through binding to TrkA and p75NTR receptors (Lazaridis et al., PLoS Biol 2011), activating prosurvival mir21 and anti-apoptotic Bcl-2 proteins, thus preventing the apoptotic loss of NGF receptor positive sensory and sympathetic neurons in NGF null mice. Phylogenetic findings on the evolution of neurotrophins, their receptors, and CYP17, the enzyme responsible for DHEA biosynthesis, combined with our data support the hypothesis that DHEA served as a phylogenetically ancient neurotrophic factor. These findings suggest that neurosteroid DHEA may act as a small molecule with NGF receptor agonist properties. However, DHEA is metabolized in vivo to sex steroids, affecting the endocrine system and increasing the risk for hormone-dependent tumors. We have recently synthesized 17-spiro analogs of DHEA with strong neurotrophic acetylcholine receptor with high affinity. Implications for Alzheimer’s disease pathology, BioL Chem 275(8), 5626–5632.

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Efficiency of selegiline loaded PLGA-b-PEG nanoparticles in crossing blood brain barrier in vivo

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Alzheimer’s disease (AD) is an irreversible and progressive neurodegenerative disease that is caused by the irreversible loss of neurons in the hippocampus and cortex regions of the brain. The deposition of the amyloid beta proteins (senile plaques) in the extracellular synaptic spaces of the neocortex is suggested to play a major role in progress of AD. The increased activity of monoamine oxidase-B (MAO-B) in AD brains was suggested to cause oxidative damage, and MAO-B inhibitors have been reported to inhibit the neuronal degeneration. Selegiline, a selective MAO-B inhibitor, known to have beneficial effects in the brain regions which are rich by dopamine receptors, however, studies based on brain targeting of selegiline are limited.

Since some recent studies showed the possible Aβ-fibril destabilizing effects of MAO inhibitors, present study was designed to (i) prepare the selective MAO-B inhibitor selegiline loaded Poly...
SW04.S16–215
Intranasal delivery of HMGB1 siRNA confers target gene knockdown and robust neuroprotection in the postischemic brain
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Non-invasive intranasal drug administration has been noted to allow direct delivery of drugs to the brain. In the present study, the therapeutic efficacy of intranasal siRNA delivery was investigated in the postischemic rat brain. FITC-labeled control siRNA was delivered intranasally in normal adult rats using e-PAM-R, a biodegradable PAMAM dendrimer, as gene carrier. Fluorescence-tagged siRNA was found in the cytoplasm and processes of neurons and of glial cells in many brain regions, including the hypothalamus, amygdala, cerebral cortex, and striatum, in 1 hour after infusion, and the FITC fluorescence was continuously detected for at least 12 hours. When siRNA for high mobility group box 1 (HMGB1), which functions as an endogenous danger molecule and aggravates inflammation, was delivered intranasally, the target gene was significantly depleted in many brain regions, including the prefrontal cortex and striatum. More importantly, intranasal delivery of HMGB1 siRNA markedly suppressed infarct volume in the postischemic rat brain (maximal reduction to 42.8 ± 5.6% at 48 hours after 60 min middle cerebral artery occlusion) and this protective effect was manifested by recoveries from neurological and behavioral deficits. These results indicate that the intranasal delivery of HMGB1 siRNA offers an efficient means of gene knockdown-mediated therapy in the ischemic brain.

SW04.S16–216
Change of hypothalamic and peripheral levels of appetite related hypothalamic neurohormones in olanzapine treated male Wistar rats
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The mechanism of weight gain due to treatment with olanzapine, a serotonin receptor antagonist, has not been fully understood. Weight gain and food intake are under the control of neuropeptides/hormones, POMC (proopiomelanocortin), CART (cocaine and amphetamine regulated transcript), AgRP (Agouti-related peptide) and NPY (neuropeptide Y) that are synthesized and secreted from the arcuate nucleus (ARC) of hypothalamus. In this study, the alteration of the ARC neuropeptide/hormone levels in rats were determined as one of the weight gain mechanisms. To understand the underlying mechanism of olanzapine induced weight gain, the drug was orally administrated to healthy male Wistar rats to analyze both the hypothalamic gene expression and peripheral levels of those candidate neuropeptides. In rats food consumption was increased and hypothalamic mRNA levels of NPY, AgRP and POMC were decreased while CART levels did not show any alteration. Consistent with the expression data, circulating levels of NPY, AgRP and α-MSH decreased significantly but CART levels were also reduced unexpectedly. In conclusion, it may be presumed that the antagonistic effect of olanzapine on the ARC neurons might be the basis for a disregulation of the neurohormones secretion which may cause weight gain during treatment.

SW04.S16–217
Atypical anti-psychotics and weight gain: risperidone induced male Wistar rats shows increased food intake and change levels of hypothalamic and circulating appetite related neurohormones
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Although the use of atypical antipsychotic drugs is successful in the treatment of schizophrenia, they cause complications in the long term use that is mainly weight gain. Patients using these drugs tend to leave the treatment primarily due to this side effect. Atypical antipsychotics’ mechanism of action is regulating the highly disrupted neurotransmitter pathways in brains of psychotic patients. Moreover, this disruption causes impairment of the neurohormone/neuropeptide pathways in different brain areas. In this study, we investigated the circulating levels of hypothalamic neuropeptides/hormones, which are related to appetite regulation; neuropeptide Y (NPY), alpha melanocyte stimulating hormone (α-MSH), cocaine and amphetamine regulated transcript (CART), Agouti-related peptide (AgRP) and plus leptin in male Wistar rats who were treated with risperidone, which is a serotonin antagonist for 4 weeks. Alterations of those candidate genes mRNA expression levels in the hypothalamus were also analyzed. Based on the hypothesis that the risperidone treatment might alter both hypothalamic and the circulating levels of those neuropeptides through the serotoninergic antagonism and it results in the weight gain. The gene expression studies showed that the mRNA expression and plasma levels of POMC, AgRP, and
NPY were decreased but CART mRNA levels were increased while their plasma levels were decreased unexpectedly. In conclusion, the serotonergic antagonism of risperidone on POMC neurons may cause increase in appetite; and hence, increased weight gain and leptin levels, even in a short term trial.

SW04.S16–218
Mechanisms of prenatal hyperhomocysteinemia neurotoxicity: the effect on the offspring
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An excessive formation of homocysteine in the methionine metabolic cycle interrupts its remethylation to methionine (methionine synthase and 5,10-methylenetetrahydrofolate reductase) and transsulfuration to cysteine (cystathionine-β-synthase), which results in hyperhomocysteinemia (HHC). Both homocysteine accumulated in neurons and the product of its spontaneous oxidation, homocystic acid, show a pronounced neurotoxic effect due to their ability to activate ionotropic and metabotropic receptors of glutamate, with which homocysteine has a structural similarity (Zieminska T et al., 2003; Boldyrev AA, 2009).

The present study was performed using an animal model of experimental prenatal HHC caused by the methionine loading of pregnant Wistar rats. It has been shown that it neurotoxically affected viability of the cerebellum neurons and cognitive ability of the offspring. The cytometric study of neurons isolated from the cerebellum of 10-11-day-old pups that developed under conditions of prenatal HHC revealed the increased levels of reactive oxygen species and necrotic cells after oxidative stress had been induced by incubation of cells with 5 mM of H2O2. An assessment of cognitive abilities of the offspring with the use of the Morris water maze test has revealed that the pups that underwent prenatal HHC demonstrated a significantly longer time of the search for a platform and a lower swimming rate, when compared to the control group, which testifies to the learning and memorizing capacities being decreased. After reaching puberty, the same rats demonstrated the disturbed hypothalamic regulation of estrous cycles manifested in no changes in the catecholamine level in hypothalamic structures responsible for the gonadotropine-releasing hormone synthesis and secretion. Besides, there was obtained data on a protective effect of melatonin and a number of peptide preparations of the pineal gland in the offspring under conditions of prenatal HHC [Arutjunyan AV et al., 2012], which allows considering them as advanced drugs to cure HHC in the pregnant women.

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SW04.S16–220
Urokinase system: the role of multidomains structure in regulation of blood vessels growth and remodeling
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The urokinase plasminogen activator (uPA) is a serine protease that converts the plasminogen in plasmin and is involved in a number of physiological processes such as angiogenesis, wound healing, vascular remodeling and carcinogenesis. The presence of multiple domains in the structure of uPA allows it to regulate cell migration, adhesion and proliferation through both proteolysis-dependent and independent mechanisms.

We have shown in endothelial and vascular smooth muscle cells the non-proteolytic mechanisms of uPA action, which include activation of signaling cascades via binding of uPA to its receptors (uPAR/CD87 through growth factor like domain, and integrin Mac-1 through kringle and proteolytic domains). Kringle domain can bind to other uPA receptors yet studied insufficiently. Proteolytic mechanisms include uPA-induced plasmin generation at the adhesion sites, the extracellular matrix degradation, the weakening of cell-cell contacts and the detachment of the leading edge of migrating cells. In addition, the proteolytic activity of uPA affects matrix proteins regulating intracellular signaling and cytoskeletal reorganization, cell adhesion and chemotaxis, and induces the increases in the expression and activation of matrix metalloproteinases. Using the recombinant mutant uPA forms and uPA neutralizing antibody, we have shown that proteolytic mechanisms are predominantly important for arterial remodeling in vivo.

Injections of uPA gene constructs to the ischemic rat heart or hindlimbs lead to the enhanced vascular- and angiogenesis. For pro-angiogenic uPA effects both its ability to bind to uPA receptor and its proteolytic activity are important. In endothelial cells, uPA increases their invasive potential, when the addition of uPA angiogenesis is limited but it is important for reparative processes and progression of diseases. In those conditions the synthesis of major angiogenic mediators, like vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), fibroblasts growth factor (FGFs), cytokines and chemokines, including IL-8, is regulated by transcription factors activated by inflammatory stimuli (Dulak et al. 2008 Circulation 117, 231–241; Florczyk et al. (2011) Free Radic Biol Med 51, 1882–1892). The special role is played by the hypoxia-inducible factors (HIFs), the master players of gene expression driven by decreased oxygen concentration (Loboda et al. 2008 Antioxid Redox Signal 10, 1767–182; Loboda et al. (2012) Vascular Pharmacol 56, 245–51). Anti-oxidant genes, like heme oxygenase-1 (HO-1) have been also demonstrated to affect significantly inflammation-driven angiogenesis, working upstream and downstream of VEGF and SDF-1 in wound healing or tumor vascularisation (Deshane et al. 2007 J Exp Med 204, 605–618; Grochot-Przeczek et al. (2009) PLoS One 4, e5803. Grochot-Przeczek et al. (2012) Clin Sci 122, 93–103). Recently, the significance of microRNAs has been also recognized in blood vessels formation. The potent regulation of microRNAs by hypoxia, HIFs and HO-1 (Kozakowska et al. (2012) Antioxid Redox Signal 16, 113–27, Jazwa et al. (2013) Cardiovasc Res 97, 115–124) indicates for the new interactions which could be be exploited for therapeutic purposes in inflammation-dependent diseases.

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Mechano-growth factor: from the mechanisms to therapeutic application

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Mechano-growth factor (MGF) is a product of alternative splicing of the insulin-like growth factor 1 (IGF-1) mRNA. MGF expression is dramatically increased in response to mechanical stimuli and tissue damage. Contrary to a generalized IGF-1 response MGF exerts a local effect and is known to stimulate myoblast proliferation and to protect neurons and cardiomyocytes from apoptosis. The prospects of MGF use for the improvement of muscle activity indicators of the aged people, patients with some forms of myodystrophy and sportsmen are widely discussed.

We aim to develop a therapy based on MGF and are exploring two possible ways of MGF delivery: administration of the recombinant protein and endogenous stimulation of MGF synthesis. Full size MGF product was expressed in S. cerevisiae, isolated, purified and characterized. The results of preclinical trials will be presented showing its high efficacy in various animal models including myocardial ischemia, cardiomyopathy, myocarditis and muscle dystrophy.

An alternative to MGF administration would be its specific induction in response to certain stimuli. However the mechanisms of induction of MGF expression are so far rather poorly understood. We investigated regulatory cascades associated with MGF induction and identified second messengers implicated in signal transduction. We also identified a number of factors that induce MGF synthesis, including stress-factors specific to muscle damage like acidification and hyperthermia, and several myofibril associated proteins that could be released during cell damage. Moreover within these proteins we were able to identify two groups of individual structural domains responsible for MGF induction and show that they signal through two distinct types of receptors.

DNA aptamers as antithrombotic therapeutic agents

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Intravascular thrombosis causes the development of myocardial infarction, ischemic stroke, and occlusions of peripheral vessels. Thrombin is a central protease of coagulation cascade. Its prothrombotic action is mediated by proteolytic transformation of fibrinogen to fibrin and activation of platelet PAR (Protease-Activated Receptors) receptors. Direct thrombin inhibitors are effective antithrombotic drugs. The goal of this study was the design of a new thrombin inhibitors based on aptamers – a new class of pharmacological substances. Aptamers are small DNA/RNA fragments which are able to interact with their protein targets with specificity and affinity comparable with those of monoclonal antibodies. New DNA-aptamer (RE31) against thrombin was constructed. Mechanism of aptamer inhibitory action is based on its interaction with thrombin exosite 1 and competition with binding of thrombin substrates – fibrinogen and PAR receptors. Unlike other thrombin inhibitors aptamer does not affect its activity towards small artificial substrates, i.e. does not interfere with its proteolytic active center. Studies of original RE31 aptamer and previously described 31TBA (Thrombin-Binding Aptamer) aptamer were performed by measuring the time of fibrin formation in blood plasma using three standard tests – thrombin time, activated partial thromboplastin time (APTT) and prothrombin time (PT). Both aptamers considerably (by more than 10-fold) increased the time of fibrin formation in all three tests. Same effects were achieved at lower RE31 concentrations in comparison with 31TBA. Life time of RE31 aptamer after injection into the blood stream did not exceed 10-15 min. In order to overcome such rapid disruption of aptamers in vivo effects we prolonged their action by using delivery system – polyelectrolyte complexes (PEC) of aptamers with protamine. Action of aptamers in such complexes were registered in vivo for up to 12 hours. APTT and PT were significantly increased in the presence of complexes in comparison with control values. Thus DNA-aptamer inhibitory activity was preserved and strongly prolonged after its incorporation into PEC.

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Antioxidant properties of apelin-12 and its structural analogue in myocardial ischemia/reperfusion injury

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Purpose: Mechanisms of attenuating myocardial I/R injury by the C-terminal residue of adipokine apelin (H-RPRLSHKGPMP-OH, A12) and its structural analogue [MeC², NLE²⁰-Thr²³]-A12 (I) remain obscure. This study was designed to examine antioxidant and free radical scavenging activity of these peptides in models of oxidative stress.

Methods: Isolated rat hearts were subjected to global ischemia followed by reperfusion with oxygenated Krebs-Henseleit buffer (KHB). They were treated with 140 mM A12 or I prior to ischemia; infusion of KHB was performed in control. Anaesthetized open-chest rats were subjected to coronary artery occlusion and recovery of coronary blood flow. Peptides were i.v injected (0.35 mmol/kg) at the onset of reperfusion; control rats received saline. The activities of Cu-Zn superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) levels were assessed in myocardial tissue. ROS generation was registered by EPR technique using a spin trap 5,5-dimethyl-pyrrroline N-oxide (DMPO) in isolated rat hearts. The activities of lactate dehydrogenase (LDH) and creatine kinase MB-fraction (CK-MB) in plasma were used as indices of cell membrane damage.

Results: Similar baseline DMPO-OH adduct concentrations were observed in myocardial effluents in all groups during preischemic
perfusion of rat hearts. In control, pour recovery of cardiac function during reperfusion was accompanied by increase in DMPO-OH adduct concentration in the effluent. Administration of peptide A12 or I improved cardiac function recovery and markedly reduced DMPO-OH formation. Both peptides increased of activity SOD and CAT, but not GSH-Px, in reperfused hearts. Treatment of rats in vivo with A12 or I reduced infarct size and LDH and CK-MB activities in plasma, prevented reduction of GSH-Px activity, and significantly augmented activity of SOD and CAT in the area at risk (AAR) by the end of reperfusion. Tissue level of lipid peroxidation product MDA in the AAR reduced to the baseline under the effect of A12 or I administration.

**Conclusion:** Cardioprotective effects of C-terminal fragment of natural adipokine A12 and its analogue I are associated with reduction of short-lived ROS generation and lipid peroxidation due to enhancement of antioxidant capacity of reperfused heart.

**SW04.S16-224**
The effect of growth hormone receptor (GHR) exon-3 polymorphism on diabetes and coronary heart disease

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**Introduction:** Diabetes mellitus (DM) and coronary heart disease (CHD) with higher prevalences throughout the world, are multifactorial diseases in which many genes and environmental factors contribute to their etiopathogenesis. The growth hormone (GH) regulates lipid, carbohydrate, protein and mineral metabolisms, and due to its importance in metabolic control in recent years studies are accelerated in determining the possible effects of GH on the etiology of DM and CHD. GH signals, via the growth hormone receptor (GHR). In humans GHR gene composed of 9 exons and has two common variants depending on the expression of exon 3 (conferred as full length, GHR-fl and deleted, GHR-d3 variants). The aim of this study was to investigate the effects of GHR exon 3 polymorphism on the development of DM or CHD, and their distribution in Turkish population.

**Materials and methods:** A total of 90 CHD and 90 DM patients, and 96 healthy controls were used to isolate the genomic DNA with salting-out procedure. The DNA samples were then analysed for the GHR exon 3 polymorphisms by multiplex PCR with locus-specific primers as previously reported.

**Results:** As expected, both CHD and DM patients showed significantly higher prevalences of traditional risk factors with respect to healthy subjects. The frequency of GHR-fl, GHR-d3 and GHR-fl/d3 genotype in control group was 43.8%, 36.5%, 19.8%; in DM group was 54.4%, 37.8% 7.8 and in CHD group was 44.4%, 45.6%, 10.0% respectively. It was seen that the frequency of heterozygous GHR-fl/d3 genotype was rare in Turkish population. On the other hand in CHD group the allelic and genotypic frequencies of GHR exon 3 polymorphism were not significantly different between study groups (p > 0.05). However in DM patients group the heterozygous genotype of GHR exon3 polymorphism (GHR-fl/d3) was significantly lower than controls (p < 0.05) and this situation was somehow valid for CHD patients group (p = 0.068).

**Discussion:** Although this report was the first study to examine the associations between GHR exon 3 variants and the development of DM or CHD, any causal role of GHR exon 3 variants in the pathogenesis of DM and CHD were found.

**SW04.S16-225**
New insight on the interplay between the urokinase and TNFalpha in MMP9 expression as a perspective in creation of new anti-invasive drugs

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Urokinase (uPA) is implicated in fibrinolysis, inflammation, angiogenesis, tumor metastasis and vascular remodeling. Urokinase acts as a factor of invasion by activating a variety of proteolytic enzymes including matrix metalloproteinases (MMPs). Attraction of monocytes into the damaged vascular tissue probably is one of the mechanisms of urokinase-mediated vascular remodeling. Monocytes in this case serve as a source of cytokines and growth factors. One of most significant factors in regulation of inflammation is necrosis factor-alpha (TNFalpha). Our previous works suggest interplay between TNFalpha and uPA. Our studies have shown that both urokinase and tumor necrosis factor-alpha (TNFalpha) induce expression of matrix metalloproteinase-9 (MMP9) in THP-1 monocytes at the level of mRNA and protein synthesis. We found that blocking of TNFalpha binding to its receptor inhibits MMP9 exerted by urokinase. Moreover, urokinase causes a brief increase in TNFalpha mRNA expression, which reached a maximum 8 hours after the addition of urokinase. We have also demonstrated that the inhibition of NFKB, the primary effector of TNFalpha, suppresses the expression of MMP9 induced by urokinase. These findings indicate participation of TNFalpha and NFKB in the MMP9 production by the action of urokinase in THP-1 monocytes. Our data suggest new insight on the perspectives of decrease in cell invasiveness by inhibition of urokinase activities.

**SW04.S16-226**
Phthalocyanines and 5-aminolevulinic acid as novel drugs for photodynamic treatment of human vascular cells

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The method of photodynamic therapy (PDT) that includes using of non-toxic dyes or photosensitizers in combination with harmless visible light has been known for over a 100 years, but only now it is becoming widely used. Originally developed as a tumor therapy, recently PDT has been proposed to be successfully applied for non-malignant disease, particularly for cardiovascular diseases. In present study we explored susceptibility of human endothelial cells (HUVEC), pericyte-like cells and macrophages to PDT using aluminium-phthalocyanine (Photosens, PS), metal-free phthalocyanine (Phthalosens, PtS) and 5-aminolevulinic acid (ALA). Dark toxicity of examined substances wasn’t detected except for 10 mM ALA which significantly reduced endothelial cell viability. Illumination of PS or PtS loaded cells (1–20 J/cm²) impaired cellular viability in dose-dependent manner. Macrophages were the most resistant to phthalocyanine-PDT despite of the great photosensitizer accumulation. HUVEC were the most susceptible one. In all cases PtS-PDT was three times more effective than PS-PDT. In contrast to phthalocyanines ALA is only a
metabolic precursor of endogenous protoporphyrin IX and doesn’t possess photosensitizing ability. Thus, cytotoxicity of ALA-PDT depends on protoporphyrin IX formation in cells. We demonstrated that pericyte-like cells tended to synthesized more PpIX than other cells while HUVEC didn’t accumulate PpIX at all. Macrophages from a part of donors produced PpIX while from other one didn’t. ALA-PDT allowed effective elimination of pericyte-like cells and some macrophages but not endothelial cells. Our results indicate that among examined photosensitizers PtS is the most effective one while ALA may allow selective elimination of pericyte-like cells. Our data are both important for understanding the non-malignant cell susceptibility to PDT and development of new therapeutic approaches for treatment and diagnostics of cardiovascular diseases.

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SW04.S16–227
Development of drugs used for therapy of duchenne muscular dystrophy: crystallization of hematopoietic prostaglandin D synthase-inhibitor complexes in space
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Duchenne muscular dystrophy (DMD) is one of the most common types of muscular dystrophy and affects approximately 1 in 3500 male births worldwide. DMD is a severe X-linked muscle disease and is characterized by progressive skeletal muscle atrophy and caused by mutations to the DMD gene which encodes the (cytoskeletal) muscle protein dystrophin. There are currently no viable treatments for this catastrophic disease. In an examination of DMD patient muscle tissues, our team found that grouped necrotic muscle fibers in these samples expressed hematopoietic prostaglandin D synthase (H-PGDS), which catalyzes the biosynthesis of prostaglandin D2 (an allergic and inflammatory mediator). The team crystallized human recombinant H-PGDS in complexes with a variety of inhibitors whose half maximal inhibitory concentrations (IC50s) were in the sub micromolar range by using the counter-diffusion method onboard the Russian Service Module and the Japanese Experimental Module ‘Kibo’ on the ISS. The three-dimensional structures of H-PGDS inhibitor complexes were determined by X-ray diffraction analysis of the microgravity-grown high-quality crystals using an intense X-ray at the SPring-8 synchrotron facility. Based on the fine structure of the inhibitor within the catalytic pocket of human H-PGDS, a novel potent inhibitor with an IC50 of 20 nM was developed. In subsequent preclinical studies, the treatment of genetically dystrophin-deficient mdx mice and DMD dogs with the novel H-PGDS inhibitor prevented the expansion of muscular necrosis and muscle atrophy without any side effects. These results indicate that H-PGDS inhibitors hold potential for the treatment of DMD patients. This study was funded in part through the High-Quality Protein Crystal Growth Experiment on JEM promoted by JAXA. The research team is grateful to the Russian Federal Space Agency and RSC Energia for the use of the Russian Service Module, and the Russian Space vehicles Progress and Soyuz for space transportation and to the U.S. ISS National Laboratory for providing access to the Commercial Generic Bioprocessing Apparatus (CGBA) hardware which provided temperature regulation for these experiments.

SW04.S16–228
CDKN2A and CDKN2BAS expression levels in patients with atherosclerosis
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Atherosclerosis is the chronic disease of arterial wall and has a multifactorial polygenic inheritance. The single nucleotide polymorphisms (SNPs) on chromosome 9p21 INK4a-ARF locus have been shown to play an important role in cardiovascular diseases. The INK4/ARF locus transcripts p16INK4a inhibits the activities of CDK4/CDK6 and arrests the progression of cell-cycle. CKIs also regulate the G1/S phase progression in VSMC and may modulate the early stages of atherosclerosis. Thus, we aimed to determine expression levels of the INK4/ARF locus genes CDKN2A and CDKN2BAS in carotid plaques and saphenous tissue samples in symptomatic and asymptomatic atherosclerosis (CA) patients. Also, the expression levels of p16INK4a protein and cell proliferation levels of tissues were determined.

The study is composed of 50 symptomatic and asymptomatic patients with CA. In carotid plaques and saphenous tissue samples gene and protein expression levels were determined by quantitative real-time PCR (qRT-PCR) and immune histochemistry (IHC) respectively.

The severity of internal carotid artery (ICA) stenosis was found to be higher in symptomatic patients (χ2 = 11.022; p = 0.001). The CDKN2A mRNA levels were significantly increased in carotid plaques compared to saphenous tissues (p = 0.009), yet the mRNA levels of CDKN2BAS were not different when we compared carotid plaques with saphenous tissues (p = 0.157). In carotid plaques of symptomatic patients CDKN2A mRNA levels were found to be higher in comparison to asymptomatic patients (p = 0.050). However, the mRNA levels of CDKN2A did not show any significant difference in saphenous tissue samples when symptomatic and asymptomatic patients were compared. None of the samples of carotid plaques and saphenous tissues showed any relation in CDKN2BAS mRNA levels between symptomatic and asymptomatic patients (p > 0.05). P16 immune (+) cells were found to be higher in carotid plaques of symptomatic patients when compared with asymptomatics (p = 0.056). Cell proliferation index was also found to be increased in carotid plaques of symptomatic patients when compared with asymptomatics (p = 0.001). This study suggests that CDKN2A transcript may affect the pathogenesis of CA and p16 protein may be associated with the subgroups of CA.

SW04.S16–229
Effects of combination treatment with amiodarone and vitamin U (methylmethionine sulfoxonium chloride) on gingiva of rats
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Amiodarone which is used for the treatment of arrhythmias, causes many side effects in all organ systems. S-Methylmethionine sulfoxonium is a derivative of the amino acid methionine and...
it is widely referred to as vitamin U (Vit U). It may protect amiodarone side effects. In literature there is no study which focuses on these substances on gingiva. In this study, we aimed to investigate and explore the effects of amiodarone and Vit U on rat gingiva. Male Sprague-Dawley rats were randomly divided into four groups. Group I; control animals receiving corn oil. Group II; control animals receiving Vit U (50 mg/kg) for 7 days orally. Group III; animals receiving 100 mg/kg amiodarone for 7 days orally. Group IV; animals receiving Vit U for 7 days orally (in the same dose and at the same time) 1 hour prior to the administration of amiodarone. All animals were fasted overnight and on the 8th day they were sacrificed under ether anesthesia. Gingiva tissue was taken from animals and homogenized in saline. Glutathione, lipid peroxidation, total protein levels, superoxide dismutase and catalase activities were determined in homogenized tissue samples. Amiodarone caused significant increase in gingival lipid peroxidation levels and in catalase activity while a significant decrease was observed in superoxide dismutase activity. Non significant decrease was also observed in GSH levels. In connection herewith, it may be suggested that the Vit U combination with amiodarone reversed these effects and such effects were significant for lipid peroxidation. These results demonstrated that administration of Vit U may have protective effects on gingiva in amiodarone treatment by decreasing oxidative stress. This study was supported by a grant from Scientific Research Project Department of Marmara University (Projects No: SAG-D- Type Project).

SW04.S16–230
Bioengineering of antimicrobial peptide arenicin analogs with improved therapeutic indices by site-directed mutagenesis
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Endogenous antimicrobial peptides (AMP) are ancient molecular factors in the evolution of innate immunity that are considered to play a key role in host defense. In recent years AMP have received widespread attention as possible alternatives to conventional antibiotics due to their rapid efficacy to a broad range of multiresistant pathogens, the membrane-lytic mode of action and the consequent low rate of resistance development. Among the most potent AMP of animal origin are peptides with β-hairpin structure stabilized by disulfide bonds. However their relatively high toxicity towards mammalian cells is an obstacle in the way of development of AMP-based therapeutics.

We have discovered a new family of cationic AMP, termed arenicins, in coelomocytes of the marine polychaeta lugworm Arenicola marina. Arenicins are small (21-residue) β-hairpin peptides stabilized by a single disulfide bond. They exhibit potent activity against Gram-positive, Gram-negative bacteria and fungi. To gain a better understanding of structure-activity relationships and search for antimicrobials having lower cytotoxicity, we designed a number of arenicin-1 recombinant analogs with amino acid substitutions. The modified arenicin analogs were obtained by site-directed mutagenesis of plasmid pET-His8-TrxL-Ar1 which had been used earlier for heterologous expression of the original peptide. The expression system is based on use of bacteriophage T7 RNA polymerase in E. coli BL21(DE3) cells. To increase yield and facilitate purification process, the recombinant peptides were obtained in the form of fusion proteins with N-terminal octahistidine tag and the modified thioredoxin A as a carrier protein. The mutagenesis technique was based on inverse PCR amplification of the whole expression plasmid. A set of mutagenic oligonucleotides annealing at the arenicin-1 coding sequence were used as sense primers, and one universal oligonucleotide specific to C-terminal part of the modified thioredoxin A acted as an antisense primer. The HindIII restriction sites were incorporated into 5′-ends of primers to facilitate PCR product recircularization by ligation using compatible cohesive ends. The most therapeutically valuable analogs were selected by comparative analysis of their antimicrobial and hemolytic activities.

The reported study was partially supported by the Federal Target Program ‘Scientific and Science-Educational Personnel of Innovative Russia’, state contract 8043.

SW04.S16–231
Streptokinase influences tissue-type plasminogen activator activity in HUVEC
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Streptokinase (Sk) is one of the most widely used thrombolytic agents of a bacterial origin [1]. There are a little data on the variety of processes that Sk induces in the organism aside of plasmin formation. Recently it has been shown that Sk caused tissue-type plasminogen activator (t-PA) activity elevation [2]. As vascular endothelial cells are thought to be the main source of plasma t-PA we estimated Sk effect on Human Umbilical Vein Endothelial Cells (HUVEC).

Cells were planted in 24 well plates in a density of 10,000 cells/cm² and were left to grow before they have reached confluence. On the fourth day before the stimulation by Sk the growth medium was changed to RPMI. Sk was added to the activation medium to the final activity of 1.5 IU/ml. t-PA activity was determined in the euglobulin fraction of activation medium in 3–240 min of incubation. The analysis was performed using S-2251 (chromogenic plasmin substrate) and fibrinogen fragment FCB-2.

It was not detected any statistically significant changes in control (without Sk) samples depending on the time of incubation. t-PA activity stood at the level of 35.7 ± 2.5 IU/ml. Three minutes after Sk addition t-PA activity tended to increase to 60 ± 3.8 IU/ml. The investigated parameter continued to rise during the entire experiment, reaching maximal value at 240 min of incubation. By this point t-PA activity amounted to 318.8 ± 18.1 IU/ml.

Conclusion: Streptokinase causes elevation of tissue-type plasminogen activator activity in Human Umbilical Vein Endothelial Cells culture.

References

SW04.S16–232
Role of ischemic preconditioning and tempol in ischemia/reperfusion injury in isolated rat heart
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Tempol is a stable piperidine nitroxide which permeates biologic membranes and behaves as a superoxide dismutase (SOD) mimic.
It reduces reactive oxygen species (ROS) production and apoptosis in cardiac cells, as well as injuries and dysfunctions induced by ischemia/reperfusion (I/R). Ischemic preconditioning (IPC) studies revealing many signaling pathways in heart protection showed to reduce injury followed by prolonged ischemia. This study aimed to investigate any protective effect of combined tempol and IPC on I/R injury in rat hearts, since the role of ROS signaling in ischemia is unknown. 56 male Wistar albino rats of 3 months old were used, by employing Langendorff isolated heart system. Six groups were formed on the basis of different time points for control, I/R and IPC groups to study in the presence or absence of tempol administration. After a stabilization period of 30 min, control groups were subjected to 130 min of continuous perfusion. I/R groups were designed as 35 min perfusion plus 35 min global ischemia and 60 min reperfusion. IPC groups were subjected to brief ischemia and reperfusion periods. Cardiodynamic parameters were recorded during the experiment. Biochemical studies were conducted on homogenized hearts. Data were statistically evaluated. End-diastolic pressure (EDP) and rate product pressure (RPP) levels were both close in control, IPC and tempol groups and almost stable during the experiment. EDP values were markedly higher, whereas RPP levels were significantly lower in I/R groups. However, the best recovery was observed in IPC group. A profound increment was observed in creatine kinase-MB levels in I/R group, while IPC, IPC+IR, Tempol and Tempol+I/R groups showed a significant decrement. With respect to lactate dehydrogenase values, a significant increment was detected in I/R and Tempol+I/R groups, but IPC, IPC+IR and Tempol groups indicated an obvious decrement compared to I/R group. When SOD results were analysed, Tempol group had lower levels compared to I/R group, while the level in other groups compared to controls. As a result, we concluded that I/R leads to damage in heart tissue. IPC improves this partially, but tempol does not exhibit a positive effect in this process.
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Stress is one of the most important factors in pathogenesis of cardiovascular diseases. In such a case it’s important to describe an influence of stress (allostasis) on different homeostasis systems, and first of all, haemostasis, pathology of which leads to such diseases as thrombosis, ischemia, insults, and heart attacks. We have studied influence of different stress factors (examinations or sport competition, etc.) on 90 healthy volunteers with different levels of heart rhythm tension (examined by heart rhythm variation method) to examine impact of stress on haemostasis. We also used the thrombodynamics test, a novel integral method to determine blood coagulation speed using the “Thromboimager-2” device (Hematological Corporation, Inc, Moscow, Russia) as described by Ataullakhanov et al. (2003; 2011; U.S. Patent). Thrombodynamics test is designed to measure in vitro spatial-temporal parameters of clot formation to study the activity of coagulation factors in blood. Lag-time, initial and stationary rates of clot formation can be recorded when using this device. The first one, time between contact of plasma to tissue factor and start of clot formation, allows to learn on quantity and activity of factor VII, factor X, TFPI and some others in blood plasma. Fibrinogen, prothrombin and protein C cause the alterations to initial speed. The changes in stationary speed represent the most common result of an involvement of the fibrinolytic or the antithrombin systems. We also examined our volunteers using the Spielberger test in respect that the personal psychological characteristics substantially impact the influence of stress factors on the physiology of men’s health. Our results show, that coagulometric and thrombodynamics data are in good agreement between themselves and with data acquired with other physiological methods. Correlation between clot formation speed and anxiety we have shown can be explained by the reaction of kallikrein-kinin system to psychological and/or physiological stress.

Reference

SW04.S16–235
Evaluation of blood coagulation, heart rhythm variation and psychological personality as novel approach to measure stress response by thrombodynamics test
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Stress is one of the most important factors in pathogenesis of cardiovascular diseases. In such a case it’s important to describe an influence of stress (allostasis) on different homeostasis systems, and first of all, haemostasis, pathology of which leads to such diseases as thrombosis, ischemia, insults, and heart attacks. We have studied influence of different stress factors (examinations or sport competition, etc.) on 90 healthy volunteers with different levels of heart rhythm tension (examined by heart rhythm variation method) to examine impact of stress on haemostasis. We also used the thrombodynamics test, a novel integral method to determine blood coagulation speed using the “Thromboimager-2” device (Hematological Corporation, Inc, Moscow, Russia) as described by Ataullakhanov et al. (2003; 2011; U.S. Patent). Thrombodynamics test is designed to measure in vitro spatial-temporal parameters of clot formation to study the activity of coagulation factors in blood. Lag-time, initial and stationary rates of clot formation can be recorded when using this device. The first one, time between contact of plasma to tissue factor and start of clot formation, allows to learn on quantity and activity of factor VII, factor X, TFPI and some others in blood plasma. Fibrinogen, prothrombin and protein C cause the alterations to initial speed. The changes in stationary speed represent the most common result of an involvement of the fibrinolytic or the antithrombin systems. We also examined our volunteers using the Spielberger test in respect that the personal psychological characteristics substantially impact the influence of stress factors on the physiology of men’s health. Our results show, that coagulometric and thrombodynamics data are in good agreement between themselves and with data acquired with other physiological methods. Correlation between clot formation speed and anxiety we have shown can be explained by the reaction of kallikrein-kinin system to psychological and/or physiological stress.

Reference

SW04.S16–236
Design of proteolytically stable cell-permeable peptide inhibitors of the myosin light chain kinase as potential antiedemic drugs
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Myosin light chain kinase (MLCK) is the key regulator of microvascular endothelial cell contraction that mediates the development of tissue edema in stressful conditions through the increased paracellular passage of protein rich blood plasma into interstitium. The inhibition of MLCK activity in endothelium is considered a perspective new approach in antiedemic therapy. Based on the backbone of peptide 18 or L-PIK (H-Arg-Lys-Tyr-Lys-Tyr-Arg-Lys-NH2; Lukas et al., 1999) that represents a modified version of MLCK autoinhibitory motif we designed and produced the family of peptides that possess various targeted modification in L-PIK structure intended to increase its half-life in human blood plasma while retaining MLCK inhibitory activity of L-PIK (modifications are in bold): H-[N-Arg]-Lys-Lys-Tyr-Lys-Tyr-Arg-D-Arg-Lys-NH2 (PIK2); H-[N-Arg]-Lys-Lys-Tyr-Lys-Tyr-Arg-Lys-Pro-Gly-Pro-OH (PIK3); H-Arg(NO2)-Lys-Lys-Tyr-Lys-Tyr-Arg-Lys-NH2 (PIK4); H-ArgΨ(CH2NH)-Lys-Lys-Tyr-Lys-Tyr-Arg-D-Arg-Lys-NH2 (PIK5); H-ArgΨ(CH2NH)-Lys-Lys-Tyr-Lys-Tyr-Arg-Lys-Pro-Gly-Pro-OH (PIK6); cyclo-[Lys-Lys-Arg-Lys-Tyr-Tyr-Arg-Lys]- (cyclo-PIK). 1H-NMR analysis shown that introduced modifications increased half-life of the most of PIK peptides in human plasma in vitro at least threefold in the following order: PIK2 > PIK3 > PIK6 > PIK4 > L-PIK. From 25% to 100% of in vitro MLCK inhibitory activity of L-PIK was retained in the novel PIK family peptides. The relative MLCK inhibitory activity of the peptides was as following PIK2 = PIK3 = L-PIK>PIK5 > PIK6 > PIK4 > cyclo-PIK. Additionally, some of the novel PIK peptides attenuated thrombin-induced endothelial monolayer hyperpermeability better than L-PIK suggesting that their capacity to penetrate in living cells and target endogenous MLCK was not impaired. Based on stability and functional performance in vitro we identified PIK2 as leading molecule for the development of anti-MLCK drugs intended for use in urgent medical conditions. In preliminary experiments PIK2 (1.5 mg/kg, i.v.) reduced LPS-induced lung edema in Wistar rats (n = 11 each in control and +PIK2) and increased animal survival about fourfold 24 hours after LPS administration. Thus, modified MLCK peptide inhibitors show promise as potential antiedemic drugs.

SW04.S16–237
Mechanisms of immunosuppression in viral infections: From retroviruses to Ebola and influenza viruses
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Immunosuppressive domains (ISD) of retroviruses were discovered at 1985 [Cianciollo et al. 1985]. Later similar domains were found in Ebola protein GP2 by Russian group of researchers [Volchkov V.E., 1992]. Identification of similar domains in placental syncytiotrophoblasts stimulated studies in this field. It was also proved that these domains, from one side, exhibit strong immunosuppressive potential, from another side, – related to pathogenic determinants of retroviruses [Schlecht-Loufa G., et al. 2010]. Fine mapping of NS1 and NS2 proteins of highly and moderately pathogenic influenza viruses lead us to the identification of ISD – sequences closely related to Ebola virus IS-domain. Sequence analysis and synthetic peptide testing revealed that these domains are similar to other viral homologues in inhibition of T-cell activation and depression of cytokine production. Fine analysis of their structure and artificial design of synthetic IS-peptides proved the concept that viral ISD target is a transmembrane receptor of TcR, identified as an antagonists of TcR α-subunit, capable to block assembling of TcR-receptor signaling complex. Yellow is highly conservative Aspartic acid residue – nucleation point of intramembrane interaction with TcR α-subunit.

Thus, many viruses, including pandemic influenza, use for their replication the same mechanism of immunosuppression leading to immunoparalysis of T-cell response.
Fighting HIV: enzyme inhibitors, lectins, and antibodies
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AIDS was an incurable disease when first identified more than 30 years ago, but remarkable progress in the development of anti-HIV drugs has completely changed the face of the epidemic. More than 20 different drugs, mainly targeting the three HIV-encoded enzymes (reverse transcriptase, protease, and integrase) are now in clinical use, in various combinations. Rapid discovery and development of these drugs depended, to a large extent, on the availability of crystal and NMR structures of the HIV enzymes and their inhibitor complexes. However, in the absence of viable HIV vaccines, development of methods for preventing infection in the first place gained in importance. Whereas topical gels have not yet been verified as viable female-controlled antiviral agents, inclusion in them of several lectins, such as cyanovirin, scytovirin, or griffithsin, holds significant promise. These lectins retain antiviral potency at nanomolar levels, are generally non-immunogenic, and can be produced relatively cheaply. Various versions of the proteins with improved properties are being developed. Another approach aimed at both prevention and curing of infection involves creation of antibodies against surface glycoproteins of HIV, gp120 and gp41. Several antibodies directed at the N-terminal helix of gp41 have been matured from synthetic libraries and their structural properties studied in crystal structures have been correlated with their ability to neutralize the virus. Progress in these structure-driven investigations promises to improve the ability to stop transmission of the virus.

Molecular dynamics study tylosin and its derivatives binding to E. coli ribosome
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By new protein biosynthesis inhibitors development tylosin and its derivatives, namely 5 O mycaminosylxylondiolide (OMT), 23 O (tret butyloxy-carbonil glycy) OMT (BocGlyOMT), 23 O (tret butyloxy-carbonil β alanyl) OMT (BocβAlaOMT), 23 O (tret butyloxy-carbonil γ amino butyryl) OMT (BocγAbuOMT), binding was studied by molecular dynamic simulation. These OMT derivatives are promissory as precursors in peptide-macrolide conjugates, which are probes for ribosome functioning examination, synthesis and new antibiotics. In 2000s atomic structures of bacterial ribosome and its complexes with antibiotics, which clarify molecular mechanisms of ribosomal antibiotics, including macrolides, action, were obtained. But structure of tylosin (tret butyloxy-carbonil γ amino butyryl) OMT was synthesized from OMT. OMT derivatives inhibitory activity was compared in system of cell-free firefly luciferase translation.

Molecular dynamic simulation study tylosin and its derivatives complexes was performed and explanation of listed macrolides inhibitory activity difference based on obtained trajectories analysis was proposed.

Calculations were performed on Moscow State University ‘Lomonosov’ supercomputer.

Design of new inhibitors uridine phosphorylases with potential therapeutic effect
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The development of innovative drugs requires knowledge of the spatial structure of pharmacological receptors – targets for these substances. Enzymes, which control vital processes of nucleotide exchange, and, in particular, pyrimidine phosphorylases could be those targets.

Processes of re-synthesis of nitrogenous bases are of particular importance for parasitic cells of humans and domestic animals. Pyrimidine phosphorylases and particularly uridine phosphorylase (UPh), are responsible for the resistance to the antimetabolite pyrimidine bases (e.g 5-fluorouracil) in cancerous cells. Recently other functions of uridine phosphorylases were identified. It should be noted that participation of UPh in the synthesis of peptidyl nucleoside antibiotics and protection of the nervous tissue in case of hypoxia makes the application of both ligands (substrates and inhibitors) UPh and the enzyme itself in the biotechnology and pharmaceutical industries prospective.

Using X-ray structure analysis there were solved and refined at atomic resolution structures of complexes of uridine phosphorylase S. typhimurium (S/UPh) physiologically-(uridine, uracil, thymidine, a phosphate anion, potassium cation) and pharmacologically-(5-fluorouracil (5-FUra), 2,2'-angidrouridine (ANU)) significant substrates [1–3]. Structures were deposited in the RCSB Protein Data Bank. 1D PDB: 1S9r, 1ZL2, 2HSW, 2HRA, 2IQ5, 2OEC, 2PQA, 2QDK, 2RF3, 3C74, 3DDO, 3DPS, 3FWP, 3NSR.

We found the place and nature of binding of the high affinity inhibitor 2,2'-anhydrouridine with the target enzyme of pathogenic bacteria UPh S. typhimurium and human cells [1, 2]. Interaction between the enzyme and the inhibitor is made by hydrogen bonds, van der Waals contacts and stacking interactions. The mechanism of inhibition of hUPPI and S/UPh by 2,2'-angidrouridine is the same. There was shown the difference in the structures of enzymes hUPPI and S/UPh in the hydrophobic site, linking the uracil component of ANU. Methods of structural crystallography and molecular modeling confirmed that the 2,2'-angidrouridine of uridine phosphorylase is a competitive inhibitor and its action is reversible. By X-ray analysis and molecular dynamics it was shown that the functionally important loop L9 in S/UPh and hUPPI changes its conformation and position in case of the ligand binding in site of the active site residues [1, 2]. The molecular dynamics method established the influence of potassium ion on the structural stabilization of molecules of uridine phosphorylases. Amino acid residues of the active site loop L9 and the initial part of the helix H8 are particularly susceptible to change. There was an increase in the density of contacts in intersubunit interactions in dimers of uridine phosphorylases in the presence of potassium ion. We conducted in silico molecular design of high-affinity inhibitors for hUPPI and for S/UPh based on 2,2'-anhydrouridine. They are 5 – or 6-substituted ANU, where saturated aliphatic chain with an aromatic group at the end serves as a functional group. For 5-substituted 2,2'-anhydro-
uridines there are differences in the type of preferred aromatic group of inhibitor (UPPI (pyridine ring)) and SUPI (imidazole ring)). This is due to the different amino acid environment of the active site of phosphorylases from different sources. For the 6-substituted 2,2'-anhydouridines there are differences in the length of the hydrocarbon chain of optimal functional group.

There were held simulation methods of molecular docking and dynamics of complex 5-FUra with uridine phosphorylases from V. cholerae [3], Y. pseudotuberculosis [4].

On the basis of structural data received by us there was held rational design of new inhibitors of uridine phosphorylases (combination virtual screening methods, the three-dimensional quantitative structure-activity relationship (3D-QSAR), pharmacophore search) based on 5-FUra. There were determined formulas of the compounds synthesized on the basis of 5-FUra, with the largest value of the constant of binding with bacterial uridine phosphorylases and human uridine phosphorylases.

The study showed that the most promising for medical use, are zwitterionic inhibitors on the basis of 5-FUra. These compounds do not bind only with the residues of uracil-binding site of the enzyme but also coordinate the phosphate anion in the active site of the target molecule. According to the results of the work there was made an application for patent number 2012140931.

References

SW04.S16-241
Preparation of antioxidant liposomes using different methods
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Liposomes are extremely small vesicles (in nanometer range) mainly composed of phospholipids organized in a bilayer. These molecules are characterized by having a hydrophilic region and a lipophilic region. Different preparation techniques such as thin-film, reverse-phase evaporation, freeze-thaw, periodic sonication are used to produce different types of liposomes.

Free radicals are molecules that are necessary for life but they are harmful for cells when their production is not controlled. During evolution of life in oxygen-rich environment oxygen derived free radicals were formed in aerobic cells. Simultaneously in all aerobic organisms an antioxidant defense system has developed against oxidative damage.

In our study, we first prepared liposomes that contained antioxidant molecules such as polyphenols, vitamin C and vitamin E, then evaluated the antioxidant activity of these liposomes. Liposomes containing vitamin E had the highest Trolox equivalent antioxidant activity. In addition, high antioxidant activity was determined in pellets when the antioxidant activity was measured after centrifuging vitamin E containing liposomes. Among the methods we have used for preparing liposomes periodic sonication was the fastest and also most advantageous because organic solvents such as methanol or chloroform are not used for preparation.

SW04.S16-242
Evaluation of the efficiency of synthesized efflux pump inhibitors on Salmonella enterica cells
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Evaluation of new pharmacological agents against bacterial pathogens is very important in increasing of drug susceptibility. There is a great potential of multidrug efflux pump inhibitors in this field. Our study was focused on the synthesis and evaluation of the efficiency of resistance-nodulation-division family efflux pump inhibitors. We used tetraphenylphosphonium and ethidium cations as the efflux pump substrates to investigate efficiency of the synthesized inhibitors on Salmonella enterica ser. Typhimurium cells. Results of our study indicated that efficiency of the inhibitors depends on the cell strain studied as well as on the outer membrane permeability. S. enterica cells overproducing AcrAB-TolC efflux system were less sensitive to the synthesized inhibitors than wild-type cells. In contrary, AcrB mutant cells were the most sensitive to the depolarizing action of the inhibitors studied. Efflux pump inhibitors led to higher accumulation of the indicator cations in the case of EDTA-permeabilized cells. Results of our experiments indicate that both, tetraphenylphosphonium and ethidium, could be used as indicators for qualitative evaluation of the inhibitory activity of synthesized compounds. However, the indicator compound used and/or conditions of the assay affect the quantitative results obtained.

SW04.S16-243
Investigation of the effect of tuberculosis drug isoxyl on selected mycobacterial epoxide hydrolases
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Tuberculosis is one of the leading causes of death by infectious disease worldwide. Its causative agent, Mycobacterium tuberculosis, has evolved a number of strategies that enable this pathogen to survive in the hostile environment of host macrophages. Several unique features of M. tuberculosis, such as its extremely resistant cell envelope, make tuberculosis very difficult to treat. Standard antitubercular treatment lasts for 6–9 months and involves using a combination of chemotherapeutics, often with minor side effects. An even greater danger comes from the emer-
gence of multidrug resistant strains of *M. tuberculosis*, which threaten to hinder the battle against tuberculosis even more. It is clear that there is a need of developing new chemotherapeutics that would simplify and shorten the therapy.

Isoxyl, a thionoic derivative is an antitubercular drug that was introduced to clinical use in the 1960s but shortly afterwards was withdrawn, mainly due to absorption issues. Nevertheless, the strong *in vitro* antimycobacterial activity of isoxyl, especially its activity against multidrug resistant strains makes this drug a valuable model for study of new therapeutic targets and development of new antimycobacterial agents. It has been shown that isoxyl targets the essential dehydratase step of mycobacterial fatty-acid synthase type II elongation cycle (Grzegorzewicz et al. 2012 *J Biol Chem* 287), however several lines of evidence indicate that this drug also inhibits other enzymes in mycobacteria, such as stearoyl-CoA Δ9-desaturase DesA3 (Phetsuksiri et al. 2003 *J Biol Chem* 278) and α/β-hydrolase fold mammalian-type epoxide hydrolases (Brown et al. 2011 *Bioorg Med Chem* 19). In this work we investigated the effect of isoxyl on the enzymatic activity of selected epoxide hydrolases from *M. tuberculosis* using cell-free experiments.

This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0441-10.

**SW04.S16–244**

**Characterization of tylosin-related macrolides – ribosome interactions by fluorescence polarization method**

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During development of new ribosome targeting drugs including ones active against resistant bacterial pathogens the binding of antibiotics to bacterial ribosomes is the first step of biological testing. Fluorescence polarization method has been described to measure the binding of fluorescently labeled erythromycin to 70S ribosomes from *Escherichia coli* and the displacement of erythromycin from these ribosomes [1]. In this study we modified and extended this method to determine the corresponding dissociation constants for tylosin-related macrolides. Fluorescent derivatives of tylosin and desmycosin containing rhodamine, fluoresceine, BODIPY FL and nitrobenzoxadiazole (NBD) as fluorescent moieties were synthesized. Binding of these compounds to 70S *E. coli* ribosomes was studied by measuring the fluorescence polarization as a function of ribosome concentration at constant concentration of a fluorescent compound. The dissociation constants for complexes of new fluorescent macrolide derivatives with bacterial ribosomes were determined.

Interactions of a series of previously synthesized tylosin and 5-O-mycaminosyltylonolide (OMT) analogues with 70S *E. coli* ribosomes were studied by the displacement of fluorescently labeled macrolides from the ribosomes. The obtained results were fitted using the exact mathematical model for competitive binding of two different ligands to the ribosome. Antibiotic affinity to the ribosome, characterized by dissociation constants, was compared to the ability of the analogues to inhibit translation of firefly luciferase mRNA *in vitro*.

New fluorescent tylosin derivatives containing BODIPY FL and NBD groups were found to be suitable for their use in tylosin-related macrolide screening assays.

This study was supported by the Russian Foundation for Basic Researches (grants 12-04-31558-mol_a, 13-04-00986-a).

**References**


**SW04.S16–245**

**Triostin A analogues with another target**

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In the last years, the chemical syntheses of (bi)cyclic (depsipeptide analogues became of interest in different research laboratories because of their potent activity as antitumors, antivirals and antibiotics; even some of them are in phase II clinical trials [1]. These chromopeptide lactones are known to be strong inhibitors of RNA synthesis and chromatin decondensation via bisintercalation into double-stranded DNA [2]. Recent synthetic efforts are mainly focused on the Triostin A and Thiocoraline scaffolds to identify novel potent lead compounds for clinical treatment. Nevertheless, none of their analogues have shown better biological activity than their parent molecule.

Herein, we designed and synthesized a small library of simplified Triostin A analogues, which are the first ones that show better antiproliferative activity against human cancer cells than the natural compound. Our analogues were evaluated against different human cancer cell lines and showed better *in vitro* activity when compared with quinoloxine antibiotics and, in some cases, with Doxorubicin.

Our drugs entered rapidly to the cells by diffusion and caspase-3/7 activation without previous loss of cell membrane integrity indicates that our compounds cause apoptosis. However, the characterization of their biophysical properties through fluorescence assays and DNase I footprinting with ‘universal’ fragments surprised us, showing that their mechanism of action is different from the quinoloxine antibiotics’ one, leading us to the discovery of novel lead compounds with an unforeseen target.

**References**


**SW04.S16–246**

**Investigation of putative ABC transporter Rv1458c/Rv1457c/Rv1456c in mycobacteria**

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One of the challenges in the fight against infectious diseases is to control tuberculosis. The causative agent of the disease in humans – *Mycobacterium tuberculosis*, is extremely resistant to adverse environmental conditions, as well as to standard antibiotics. This resistance is mainly due to structurally unique mycobacterial cell wall, which gives the bacterium enormous robustness.

The structures of the cell wall components, as well as several enzymes involved in their biosynthesis, are relatively well described. However, there is only minor evidence concerning the transport of the cell wall intermediates across the plasma membrane. The gene *rv1459c* encoding glycosyltransferase suggested to be involved in the biosynthesis of the important cell wall poly-
saccharide – mannan, is located in the vicinity of the genes \( rvl453c, rvl457c \) and \( rvl456c \) encoding nucleotide binding domain and transmembrane domains of the putative ABC transporter. Information about the function of this ABC transporter, likely to be participating in the transfer of the cell wall metabolic intermediates across the plasma membrane, is still missing.

We tried to address this question by construction and phenotypic characterization of the strains of the non-pathogenic model mycobacterium – \textit{Mycobacterium smegmatis}, carrying disrupted genes for transmembrane domains of this transporter. Analyses of the cell wall lipids and polysaccharides of constructed strains shed light on the function of the studied ABC transporter in mycobacteria.

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**SW04.S16–248**

The influence of mutations in C-terminal domain of HIV-1 integrase on its activity and interaction with HIV-1 RT

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Reverse transcriptase (RT) and integrase (IN) catalyze the most important reactions at the earlier steps of the retroviral replication cycle. RT produces the synthesis of the DNA copy of the viral genomic RNA, and then IN integrates this DNA into the host cell genome. On the assumption of the retroviral replication mechanism, RT and IN should interact. In vitro experiments show that IN and RT bind one to another, and domains responsible for binding were identified in both proteins. However, data concerning the mutual influence of these enzymes on their catalytic activity are too limited and conflicting till now.

We prepared several IN samples containing substitutions in the C-terminal domain (CTD), which is both necessary and sufficient for binding RT. IN mutants K258A, W243E and V250E are shown to inhibit reverse transcription within HIV-1 infected cells. We also prepared integrases containing mutations R228A, D229A, R231A, D232A, K236A in the CTD, but not in the region of 242–258, which is shown to be important for IN-RT interactions, and IN with C130S mutation in the catalytic domain.

All mutants were tested for their catalytic activity. It was established that the replacement W243E almost completely inhibits the IN activity. The IN catalytic activity was also significantly decreased by mutations R228A and K258A; mutations D229A, D232A, and K236A reduced the effectiveness of the enzyme in half, and the mutation D231A increased it. The direct physical interaction between IN mutants and RT was studied in GST pulldown assay using purified proteins. The effect of all the mutants obtained on the RNase, DNA- and RNA-dependent DNA polymerase activities of RT was also studied. No significant effect on the efficiency of DNA/RNA substrate hydrolysis was detected for any of the IN samples. In the case of DNA- and RNA-dependent DNA synthesis a slight inhibitory effect of all samples except IN mutants R228A, W243E, V250E, and K258A was revealed.

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**SW04.S16–249**

Purification and function of mycobacterial WecA protein

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Tuberculosis remains one of the deadliest infectious diseases world-wide. Its causative agent, \textit{Mycobacterium tuberculosis}, is a difficult pathogen due to its specific features, which include the unique cell wall with the highly impermeable outer membrane. The key structure of the mycobacterial cell wall is the covalently linked core comprised of peptidoglycan, heteropolysaccharide arabinogalactan and the long chain fatty acids, mycolates, which form the inner layer of the outer membrane. Biosynthesis of the constituents of the mycobacterial cell wall has been in the center of the attention of many investigators for a long time, since it is the target of several drugs used for the treatment of tuberculosis. We focused on characterization of the enzyme initiating galactan biosynthesis. This polymer is built on a lipid carrier and the whole process starts with the attachment of \( N \)-acetylglucosamine-1-phosphate from UDP-\( N \)-acetylglucosamine to decaprenyl phosphate, followed by the addition of rhamnose from TDP-Rha. The product of these reactions, decaprenyl-P-P-GlcNAc-Rha then serves as the substrate for polymerization of galactose. Mycobacterial protein Rv1302 was proposed to catalyze the decaprenyl-phosphate \( N \)-acetylglucosamine-1-phosphate transferase reaction based on its similarity to proteins encoded by \textit{wecA} genes in \textit{E. coli}, catalyzing similar reaction. However, its biochemical characterization is still missing. Here we present prepa-ration of the \textit{M. smegmatis} and \textit{E. coli} strains producing recombinant mycobacterial WecA proteins from \textit{M. tuberculosis} H37Rv (Rv1302) and \textit{M. smegmatis} mc\(^{\text{2}}\)155 (MSMEG\_4947). Purification of the mycobacterial WecA from these strains allowed us for the first time to prove its enzymatic activity in vi-tro. We show that the enzyme is inhibited by tunicamycin and thus further investigation of this key enzyme in the cell wall biosynthesis might bring important information for the development of the drugs against tuberculosis.

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**SW04.S16–249**

Dimers of chloramphenicol as alternative antibacterials

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Chloramphenicol (CAM) is a broad spectrum antibiotic, which inhibits protein synthesis in prokaryotes, interacting with the large ribosomal subunit. Analysis of ribosome’s crystals from Halocurla marismortui showed that CAM binds at the exit tunnel. However, recent crystallographic studies in Escherichia coli confirmed kinetic results, according to which the antibiotic binds to the catalytic center of the ribosome. Based on these crystallographic data, we decided to design and synthesize dimers of CAM capable in binding to both sites. \textit{In silico} analysis revealed the ideal distance between the two molecules of the CAM in dimers, allowing simultaneous binding of CAM-dimer to the cat-
alytic center and to the exit tunnel. Namely, the distance between the two nitrogen atoms of CAM was found to be equal to 9.8 Å, corresponding to 6–7 successive single bonds C. Taking these data into account, we synthesized two CAM-dimers, Mag234 and Mag244, in which the two CAM molecules were separated via a diacyl linker of 6 and 8 carbon atoms, respectively. The inhibitory activity of mag234 and mag244 on peptide-bond formation was found lower ($K_i = 1.8$ and 2.1 µM, respectively) than those exhibited by the parent compound ($K_i = 0.88$ µM). The reduced inhibitory potency of mag234 and mag244 is probably related to the length and flexibility of their linker. Evidently, mag244, whose linker exceeds the limit of 9.8 Å, shows worse inhibitory activity, compared with CAM. Therefore, optimization of the linker properties appears to be a prerequisite in preparing dimers with improved activity.

**SW04.S16–250**

**Singlet oxygen effects on lipid membranes: Implication on viral fusion inhibitors**

**mechanism of action**

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Targeting membranes of enveloped viruses represents an exciting new paradigm to explore regarding the development of broad-spectrum antivirals. It was recently reported that a new aryl methyldi-ene rhodanine derivative, named LJ001, and oxazolidine-2,4-dithione [1], named JL103 [2], act on the viral membrane, avoiding its fusion with the target cell membrane. The aim of the present work was to study the interactions of both active compounds (LJ001 and JL103) and LJ025 (an inactive analog used as negative control) with biological membrane models, in order to clarify the mechanism of action of these new enveloped virus entry inhibitors. Fluorescence spectroscopy was used to quantify the partition and determine the location of the molecules on membranes, concluding that the three molecules are able to interact with lipid membranes. However, JL103 has a much more interfacial location. The ability of the compounds to produce reactive oxygen molecules in the membrane was tested by using DMA, which reacts selectively with singlet oxygen to form the non-fluorescent 9,10-endoperoxide. The changes that these molecules are able to promote on the lipid packing and fluidity of lipid membranes were assessed by surface pressure, DPH and TMA-DPH fluorescence anisotropy and Laurdan generalized polarization measurements. Finally the ability to impair membrane fusion was evaluated by Förster resonance energy transfer (FRET). Our results indicate that the production of singlet oxygen by LJ001 and JL103 are able to induce several changes on membrane properties, specially related with a decrease on its fluidity, concomitant with an increase on the order on the polar head groups region, promoting a synergistic effect between membrane curvature (or its inability to change) and fluidity, resulting in an inhibition of the formation of the fusion pore necessary for cell infection.

**References**


**SW04.S16–251**

**Rational design of apoptosis signal-regulating kinase 1 inhibitors**

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Increased activity of apoptosis signal-regulating kinase 1 (ASK1) is associated with several pathologies, including neurodegenerative, cardiovascular and liver diseases. The inhibitors of ASK1 may become important compounds for pharmaceutical application.

To discover the protein kinase ASK1 inhibitors we have performed screening program, using both in silico and in vitro approaches. AutoDock and DOCK software were used to conduct receptor-ligand flexible docking. The best-scored compounds of different chemical classes were taken for the kinase assay analysis.

In vitro observations revealed that derivatives of 2-Thioxo-thiazolidin-4-one exhibited inhibitory activity towards ASK1. The most active compound inhibited ASK1 with IC_{50} = 2 mM. Then, in-depth study of this chemical class was performed using the pre-selected library of 2-thioxo-thiazolidin-4-one derivatives. Ten best-scored compounds were taken for the kinase assay analysis. Compound 2-[5-[3(4-Dichloro-phenyl)-furan-2-ylmethyl-ene]-4-oxo-2-thioxo-thiazolidin-3-yl]-propanoic acid (PFTA-1) inhibited ASK1 with an IC_{50} = 650 nM. Our preliminary selectivity studies demonstrated that this compound seems to be selective inhibitor of ASK1. In silico analysis of the complexes of ASK1 with compounds indicated, that the peculiarity of the PFTA-1 in the comparison to other nine inactive derivatives is its ability to bind simultaneously to the part of kinase domain known as ‘hinge region’ and the phosphate-binding region of the ATP-binding cleft.

The core structure of PFTA-1 was used for developing more potent and selective inhibitors of ASK1. A series of derivatives has been synthesized and evaluated in vitro towards human protein kinase ASK1. It was revealed that the most active compounds inhibit ASK1 with IC_{50} of 200 nM. Structure-activity relationships of 33 compounds have been studied and binding mode of this chemical class has been predicted.

**SW04.S16–252**

**New carbocyclic uracil derivatives as potential antiviral and antibacterial agents**


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**Background:** Carbocyclic nucleosides are analogues of the naturally occurring nucleosides that possess a methylene group in place of the furanose oxygen atom in the sugar ring. Potent antivirals such as Lobucavir, Abacavir and Entecavir are important members of this class of compound in the field of medicinal chemistry.

Recently, 5-substituted pyrimidine nucleosides were observed to have activity against *M. tuberculosis*, *M. bovis* and *M. avium*. Specifically several 5-alkynyl, 5-phenylalkynyl, and 5-pyridylalkynyl derivatives of pyrimidine nucleosides showed noticeable antiviral activity. 5-Alkynyl carbocyclic 5’-noruridine analogues were synthesized in our laboratory and have proven to be inhibitors of *M. tuberculosis* growth. From the other hand several different types of substituted pyrimidine derivatives have shown...
remarkable anti-HIV activity. In an attempt to further explore therapeutic potential, a new type of carbocyclic hybrid nucleoside was designed based on two leads—one, the effective anti-TB activity exhibited by uridine derivatives, and two, the observation that a series of substituted uracils proved to be potent non-nucleoside inhibitors of RT-HIV.

**Methods:** The synthesis of carbocyclic uracil derivatives was accomplished using previously described method. The anti-TB activities of the 5-substituted carbo-nucleosides were then evaluated against *M. tuberculosis* H37Rv (wild H37Rv and multi drug resistant MS-115 strains) using the BACTEC assay. The anti-HIV properties of the target compounds were also examined in RT HIV-1 (wild and non-nucleoside resistant strains).

**Results:** Some of tested were shown to completely inhibit the growth of *M. tuberculosis* culture at a concentration of 10−40 mgk/ml. Moderate inhibition RT-HIV-1 was observed for N3, 4'-substituted carbocyclic uracil derivatives with Ki values of 8−50 mM.

**Conclusion:** Inhibitory activity was observed for a series of 5'-norcarbocyclic uracil derivatives against *M. tuberculosis*. In addition, a series of N3, 4'-substituted carbocyclic uracil derivatives proved to be moderate NNRTI. Further optimizations of their structures and additional antiviral and antibacterial studies are currently underway.

**SW04.S16-253**

**Anti-biofilm activity of maghemite nanoparticles coated with dextran**

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The iron oxide nanoparticles coated with dextran (DIO-NPs) were synthesized by an adapted co-precipitation method [1–2]. The DIO-NPs prepared under simple adapted chemical method have a particle size considerably smaller than that reported in the literature. The aim of this study was to evaluate their antibacterial activity against biofilm embedded cells of the Gram-negative *Pseudomonas aeruginosa* and of the Gram-positive *Enterococcus faecalis* bacterial strains, by using culture based methods. The anti-biofilm activity of the tested compounds was tested by the microtiter method. The tested nanoparticles exhibited a remarkable anti-biofilm effect manifested till very low concentrations, i.e. 0.01 μg/ml. Concerning the antimicrobial activity on cell growth in biofilms, quantified by measuring the absorbance of the adherent cells colored suspension at 492 nm, the DIOM-NPs presented an inhibitory effect on the ability of the Gram-negative *P. aeruginosa* and the Gram-positive *E. faecalis* strains to develop biofilms. The effect of DIOM-NPs on the planktonic microbial growth observed by the absorbance measurements at 620 nm was reported in Iconaru et al., Nanoscale Res Lett [3].

The specific antimicrobial activity revealed by the qualitative assay demonstrates that our compounds are interacting differently with the microbial targets, probably due to the differences in the microbial wall structures. The Gram-negative bacteria possess an outer membrane with porins and a unique periplasmic space not found in Gram-positive bacteria, favoring the internalization of nanoparticles and their intracellular accumulation. The tested DIO-NPs seemed to exhibit a better bactericidal effect on the Gram-negative strains, as compared with the Gram-positive ones, accounting for the hypothesis that they cause changes in bacterial membrane permeability, affecting the physiology and finally, the cell viability [4].

Our results showed that the tested DIO-NPs interact differently with the microbial cells in different growth states, i.e. suspension versus biofilm embedded cells, demonstrating the specificity of the molecular interactions established between the microbial cells and the tested nanoparticles. The anti-biofilm activity was superior to that antimicrobial one in the case of these nanoparticles, so they could be successfully used for further development of novel antimicrobial materials or strategies for fighting medical biofilms.

**References**


**SW04.S16-254**

**Antimicrobial activity of silver doped hydroxyapatite thin films**

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This research focuses on understanding the antimicrobial activity of silver doped hydroxyapatite thin films (Ag:HAp-TFs). Silver-doped hydroxyapatitate thin films on pure Si disks were developed. This films were made by thermal evaporation technique from Ca10xAgx(PO4)6(OH)2 with xAg = 0.5. Commercially pure Si disks were used as substrates. For evaporation in medium vacuum (p ~ 8 × 10−5 torr), a wolfram boat was used, and the intensity of the maximum current through the boat was I max = 40 A for t ~ 5 s. The The bactericidal effect against common Gram-positive and Gram-negative bacteria has been also investigated. Elemental maps for the samples prepared with xAg = 0.5 are also shown. The spectrum and images confirmed the presence of silver in the samples. The EDAX spectrum of Ag:HAp confirms the presence of calcium (Ca), phosphor (P), oxygen (O), and silver (Ag). The observation of the chemical composition of the coatings with GDOES measurements gives information on the distribution of the elements throughout the film. They show that a substantial Ag content has been deposited in the films. The XPS spectra revealed the presence of a material composed mainly of phosphate, calcium, oxygen, hydrogen and silver.

This study showed that Ag:HAp-TFs with xAg = 0.5 presented a good antimicrobial activity against *Staphylococcus aureus* (ATCC 6538) and Escherichia coli (ATCC 25922). The antimicrobial activity of Ag:HAp-TFs was tested using the standard microdilution method. *Staphylococcus aureus* (ATCC 6538) and Escherichia coli (ATCC 25922) were grown on LB agar broth with Ag:HAp-TFs (xAg = 0. 5). Yeast extract agar plates were incubated for 24 hours at 37°C and the obtained colony forming units (CFU) were visually counted. The Ag:HAp-TFs (xAg = 0. 5) show strong antibacterial activity. The in vitro bacterial adhesion study indicated a significantly reduced number of *E. coli* and *S. aureus* on Ag:HAp-TFs (xAg = 0. 5). The results of this study...
clearly demonstrated that the Ag:HAp-Tfs (xAg = 0.5) inhibited growth and multiplication of the tested gram-positive and gram-negative bacteria. Ag:HAp-Tfs (xAg = 0.5) exhibited an excellent antibacterial performance against both multiresistant bacteria such as *S. aureus* and *E. coli*. These data suggest that development of novel Ag:HAp-Tfs is a promising material with the antibacterial properties that may be used for covering the surfaces of ambulatory and other medical devices.

### SW04.S16–255

**The investigation of antibacterial effects of various 4-aryl substituted coumarin derivatives**

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Coumarins are naturally occurring compounds which are widely spread in plants, microorganisms and some animal species. Due to their remarkable array of biological activities, usually associated to low toxicity, form an elite class of compounds which occupy a special role in nature. Coumarins have diverse biological and pharmacological effects including anticoagulant, anticancer, antiviral, and cholesterol lowering.

The problem of antimicrobial resistance is a serious public health concern with economic, social and political implications. Infections caused by resistant bacteria are not only more severe and require more complex treatments, but they are also significantly more expensive to diagnose and to treat. Due to the rapidly increasing occurrence of resistance to existing antibacterial agents, it is necessary to develop new antibiotics in order to maintain a pool of effective drugs at all times. In the development of newer antimicrobials, coumarins have been identified as target specific anti-bacterial agents with growth inhibitory potential particularly against Gram-positive species.

In the present work, we have prepared five 4-aryl coumarin derivatives (7,8-dihydroxy-4(3',4'-dimethoxyphenyl)coumarin, 6,7-dimethoxy-4(3',4'-dimethoxyphenyl)coumarin, 6,7-dihydroxy-4(3',4'-dimethoxyphenyl)coumarin, 7,8-dihydroxy-4(3',4'-dihydroxyphenyl)coumarin and 6,7-dihydroxy-4(3',4'-dihydroxyphenyl)coumarin) and their antibacterial activities were tested against both Gram-positive (*E. coli*) and Gram-negative (*S. aureus*) bacteria. Resuscitation of bacterial strains was carried out in 10 ml BH Broth incubated overnight at 37°C. All broths were then incubated statically at the aforementioned temperatures for each microorganism, for 18–24 hours to guarantee that all cells were in the stationary phase. Susceptibility of the test organism to the extract was determined by employing the standard disk diffusion technique. DMSO used as solvent to dissolve coumarin derivatives and therefore used as negative control, ampicillin and kanamycin used as positive control. It was found that the test compounds containing dihydroxyl substituents have moderate to high antibacterial activity on *S. aureus* while compounds with dimethoxy substituents show very low antibacterial activity. From these results it may be concluded that because of their high antibacterial activities and probable low toxicities, dihydroxy-4-aryl-coumarin derivatives are promising and potential scaffold for designing novel antibacterial agents.

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### SW04.S16–256

**Cobalt bis(dicarbollide) derivatives as modulators of enzyme activity**

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In the last decade, boron cluster derivatives are intensively studied as modulators of enzyme activity. Thus, we designed, prepared, and tested a small library of cobalt bis(dicarbollide) derivatives for isoform-selective nitric oxide synthase binding that causes activation or inhibition of the nitric oxide synthesis. The design applies rigid and nontoxic boron clusters, previously studied for boron neutron capture therapy. Based on the concept of creating a hydrophobic analog of the natural substrate, we began with a basic boron cluster system and added a positively charged moiety to its periphery, providing hydrophobic and non-classical hydrogen bonding interactions with the proteins. Several of these compounds show efficacy for inhibition of NO synthesis and differential inhibition of the various nitric oxide synthase isoforms. Direct utilization of boron cluster derivatives is complicated due to their low solubility and self-assembling in water, thus we tested also their complexes with biocompatible excipients.

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### SW04.S16–257

**Vitalang-2: the novel antiviral agent**

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We developed a simple, easily scalable, environmentally friendly and virtually waste-free method for isolation of soapy amphiphilic high-polymer RNA from dry baker’s yeast, using sodium oleate. This RNA compound (preliminary commercial product name – Vitalang-2) is capable of easily crossing the biological membranes and efficiently induces biosynthesis of interferon-γ. The current presentation summarizes results of a study of the antiviral activity of the Vitalang-2 in an *in vivo* model utilizing highly pathogenic avian influenza A/chicken/Kurgan/05/2005 (H5N1) virus and extromelia K-1 (mouse pox) virus.

Intramuscular administration of the compound into mice at escalating doses (0.1; 0.5 and 1.0 mg/kg body weight), post-infection, induces some degree of protection (9.5%) in outbred albino mice from the lethal dose (10 LD50) of avian influenza A/H5N1 virus. Protection from 10 LD50 of the mouse pox virus was more pronounced, ranging from 28.6% to 47.6% depending on the dose of the Vitalang-2 compound administered.

Introduction of Vitalang-2 in the prophylactic scheme (i.e. prior to infection) results in even more significant protection of mice against infection with 10 LD50 of avian influenza A/H5N1 virus and extromelia. The protective effect of the Vitalang-2 compound was similar to that of the well-known commercial influenza compound oseltamivir (Tamiflu), while the Vitalang-2 was administered at 25 times lower cumulative dose. The protective effect of the Vitalang-2 displayed a pronounced dose-dependent character: out of three doses tested (0.1; 0.5 and 1.0 mg/kg body weight) the most significant effect is observed at the maximal
dose of 1.0 mg/kg. In this case the percentage of surviving animals in the group from the prophylactic scheme is 38.1% and 61.9% versus 0% and 19.1% in the control group (for infection with influenza virus and ectromelia, respectively).

Vitalang-2 has successfully passed the pre-clinical and clinical trials for veterinary viral diarrhea – mucosal disease in cattle. Based on the body of data obtained to date, this compound has high potential to become a next generation anti-viral agent.

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SW04.S16–258
Metabolism of 9-norbornyl-6-chloropurine – a novel antiviral and antileukemic agent
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The discovery of the antibiotic and antitumor activity of the natural carbocyclic nucleosides aristomycin and neplanocin A stimulated search for novel carbocyclic analogues with therapeutic potential. Moreover, carbocyclic nucleosides possess high metabolic stability toward phosphorylases and hydrolases caused by replacement of methylene group with oxygenate atom in carbohydrate ring. Here we present novel nucleoside analogue, 9-norbornyl-6-chloropurine (NCP), with a potential as an antiviral and antileukemic agent. To understand the mechanism of action, we first explored metabolic fate of the NCP. The metabolism was first explored metabolic fate of the NCP. The metabolism was

SW04.S16–259
Biochemical characterization of acyltransferase MSMEG_2934 from Mycobacterium smegmatis
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Tuberculosis remains one of the leading reasons of death caused by an infectious agent worldwide. Highly glycosylated cell surface of the pathogen causing the disease – Mycobacterium tuberculosis, plays a crucial role in pathogenesis of tuberculosis. Phosphatidylinositol mannosides (PIMs) are essential structural components of the mycobacterial cell envelope. They are involved in host-pathogen interactions during the infection and serve as a basis for the biosynthesis of other unique molecules with immunomodulatory properties, lipoarabinomannan and lipomannan.

The backbone of PIM is formed of 1,2-diacyl-sn-glycerol-3-phospho-1-D-myo-inositol (PI) decorated with one to six mannosides and one or two additional acyl groups. It has been shown that the degree of mannosylation and acylation could play a role in regulation of signaling processes through human receptors. Build-up of PIMs in M. tuberculosis is initiated by the action of two mannosyltransferases, PimA and PimB, and by the acyltransferase Rv2611c. PimA catalyses the transfer of mannosyl residue from GDP-mannose to the 2-position of the myo-inositol ring of PI and the phosphatidylinositol monomannoside (PIM₁) is formed. PIM₁ is further modified by the addition of the second mannoside to the 6-position of myo-inositol of PIM₁ by PimB forming PIM₂. In the crude cell free assays performed with mycobacterial membrane fractions, both PIM₁ and PIM₂ were shown to be acylated at 6-position of the 2-linked mannosyl residue by acyltransferase Rv2611c to form Ac₁PIM₁ and Ac₂PIM₂, respectively [1], but the latter substrate was proposed to be preferred [2]. Closer investigation of Rv2611c, including examination of its substrate specificity was precluded due to the lack of the pure protein. In this work we present cloning of MSMEG_2934, the ortholog of Rv2611c from the non-pathogenic model organism M. smegmatis mc²155, and isolation of the recombinant protein, which allowed not only confirmation of its in vitro acyltransferase activity, but also its further biochemical characterization.

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References

SW04.S16–260
Distributed informational regulatory influences (DIRI) – a new concept of drug design
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At the top level of regulation the communication in multicular organisms is orchestrated by the informational framework (IF), unique for each organism. An informational exchange at the next levels is realized as networks of soluble mediators, supramolecular complexes and extracellular vesicles. Disturbances in IF that cannot be compensated inside the system evoke extreme reorganization of IF, which in turn is the main cause of chronic disease. Changes in IF involve dysfunction of many parts of bioregulatory network. Under this situation a strong impact of target-designed monofunctional drug at only one point of this network can negatively influence meta-stability of other parts of regulatory system within a diseased organism. An alternative approach how to manage a pathology process is based on complex and smooth intervention into the regulatory network. According to the concept of Distributed Informational Regulatory Influences (DIRI), proposed by us, more successful and safe drug should be able to interact with many targets and evoke positive response in different parts of the regulatory network and thus trigger hidden mechanisms fighting against pathologic processes. In general, these multifunctional compounds will help the human body to restore IF normal state. In order to effectively switch on the protective mechanisms that are downregulated under pathology, the molecule of the multifunctional drug should contain fragments of endogenous bioregulators (like lipids, peptides, gasotransmitters, etc.) properly connected using some specific topological rules, avoiding use of bioincompatible linkers. Substances, bearing prostaglandin, its glycerol ester and nitric oxide in one structure, demonstrate superior activity as bronchodilators or microcirculation enhancers. Peptolipins that incorporate fragments of bioactive lipids and peptides act as potent neuroprotective agents. The
DIRE concept is also applicable to well-known drugs, e.g. non-steroidal anti-inflammatory drugs, to convert them into real multifunctional compounds with increased efficiency and safety for patient.

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**SW04.S16–262**

**Hepatitis B escape mutants among patients with different types of chronic hepatitis B infection**

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Hepatitis B virus (HBV) infection is an inflammatory liver disease that affects more than 2 billion people around the world. Extreme variability of HBV genome leads to the generation of new viral variants with mutations in the S gene which impair hepatitis B surface antigen (HBsAg) detection and also permits the virus to escape immune system especially in persons exposed to immunosuppressives or vaccines (escape mutants). The aim of this study was to determine the effects of genotypes, serotypes, serological profiles and S gene mutations in patients chronically infected with HBV considering duration of treatment and illness. Two groups of patients were selected; group I – cirrhosis or hepatocellular carcinoma, group II – chronic active hepatitis with/without therapy. Amplification of target HBV-DNA region were carried out by nested polymerase chain reaction (PCR) using selected primers allowing the distinction of HBV genotypes. The products of nested PCR were sequenced, using the second-round primers. The obtained sequences were submitted to the Blast program in order to determine their similarity to other HBV strains deposited in GenBank. The expected distribution of genotypes was obtained: that most of the variants belonged to genotype D (82.6%), mostly subgenotypes D3 and D2, and genotype A (15.2%), exclusively subgenotype A2 while the remaining samples belonged either to genotype F or mixed genotypes D, serotype distribution were mostly ayw3 (30%), ayw2 (41.3%) and adw2 (15.2%) which is in the correlation with the obtained genotypes. The frequency of S gene mutations in group I had higher rate in the major hydrophilic region (MHR) (44.2%) comparing to group II (32.7%). In group I, in addition to 45 previously described mutations two new mutations were identified (G71N and N206Y). In group II, 18 previously described mutations have been found. Mutations in the S gene correspond to the severity of the disease while numerous mutations in S gene outside the MHR region warn about the risk of the emergence of vaccine escape mutants. Genotyping of chronic HBV infections could help to identify patients with risk of disease progression and to determine the optimal therapy.
The chemo-enzymatic synthesis of clofarabine and related nucleosides: the role of electronic and stereochemical factors of substrates in reactions catalyzed by \textit{E. coli} nucleoside phosphorylases

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In our previous works, we studied (i) the chemical synthesis of a-D-arabinofuranose-1-phosphate (a-Ara-1P) and its use as a substrate of the enzymatic synthesis of purine and pyrimidine nucleosides, and (ii) the one-pot cascade transformation of D-ribose and 2-deoxy-D-ribose into nucleosides (Synthesis 2011, (10), 1555; Mendeleev Communication 2011, 21, 57). In continuation of these studies, we investigated here the chemical synthesis of 2-deoxy-2-fluoro-a-D-arabinofuranose-1-phosphate (a\textsuperscript{-2F}-Ara-1P) and its substrate properties for the recombinant \textit{E. coli} nucleoside phosphorylases, as well as the cascade transformation of 2-deoxy-2-fluoro-D-arabinose, D-arabinose, D-xylose and D-ribose into the corresponding b-D-pentofuranosides of 2-chloroadenine.

The chemical synthesis of a\textsuperscript{-2F}-Ara-1P is described and its use as a universal glycosylating agent for the nucleoside synthesis is studied in comparison with a-Ara-1P. It was found that the phosphate a\textsuperscript{-2F}-Ara-1P, like a-Ara-1P, is a good substrate of the recombinant \textit{E. coli} purine nucleoside phosphorylase (PNP) and can be used for the synthesis of a number of purine nucleosides (e.g., Clofarabine and related nucleosides of 2,6-diaminopurine and hypoxanthine) as well as base modified derivatives [e.g., 5-aza-7-deaza-9-(2-deoxy-2-fluoro-b-D-arabinofuranosyl)guanine]. Unexpectedly, the phosphate a\textsuperscript{-2F}-Ara-1P, unlike a-Ara-1P, showed no substrate activity for the recombinant \textit{E. coli} uridine phosphorylase (UP); both phosphates devoid substrate activity towards the recombinant \textit{E. coli} thymidine phosphorylase (TP).

As expected, D-ribose was the best substrate in a cascade transformation consecutively in D-ribose-5-phosphate (catalyzed by \textit{E. coli} ribokinase), the latter in a-D-ribofuranose-1-phosphate revealed the differences in rates of the formation of inosine and allopurinol ribose along with high yields of both nucleosides (ca. 80% after 5 min and ca. 95% after 3 hours, respectively) witnessing to non-critical role of the N\textsuperscript{2}-nitrogen atom in the binding and activation of heterocyclic substrate in the synthesis of nucleosides catalyzed by the native \textit{E. coli} PNP. Replacement of the native PNP with the mutated Ser90Ala species resulted in (i) the slowing down of the rate of the inosine formation (35% yield of inosine after 1 hour; an equilibrated ca. 2:8 mixture of base — nucleoside after 24 hours; ca. 80% yield) implying the moderate contribution of the Ser90 residue of the catalytic center of the native \textit{E. coli} PNP in the binding of a-D-Rib-1P, and (ii) dramatic reduction of the rate of the allopurinol ribose formation attaining a ca. 20% yield after 48 hours pointing to an unique importance of Ser90 residue of the native \textit{E. coli} PNP in the binding and activation of allopurinol as well as other 8-aza-7-deazapurines (especially 2-amino-8-aza-6-chloro-7-deazapurine) in the enzymatic synthesis of their nucleosides.

To gain further insight into the role of the Ser90 residue of \textit{E. coli} PNP in the nucleoside synthesis, the glycosylation reactions of selected natural and modified purine bases were studied and the results will be presented.

Disulfide-containing drug glutoxim modulates Na\textsuperscript{+} transport in frog skin

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Frog skin is a classical model object to study the ion transport mechanisms across biomembranes. Transepithelial Na\textsuperscript{+} transport is a complex system that creates and maintains electrolytic and water homeostasis. Various protein components of this system may be the targets for oxidative stress.
Recently, new disulfide-containing agents with d-metals as nanoadditives, altering cell redox state, have been widely used in clinical practice. Thus, the drug glutoxim® (oxidized glutathione disodium salt with Pt nanoaddition, PHARMA-VAM, Moscow) is widely applied as an immunomodulator and hemostimulator for the therapy of bacterial and viral infection, psoriasis and the radio- and chemotherapy of oncological diseases. However, molecular mechanisms of Na+ transport regulation by glutoxim are still unclear.

Using voltage-clamp technique we have shown for the first time that the Na+ transport in frog skin is modulated by glutoxim. Application of 100 μg/ml glutoxim to the skin apical surface inhibited Na+ transport. At the same time, when the agent was added from the basolateral side of the skin, glutoxim mimicked the effect of insulin and stimulated transepithelial Na+ transport.

It is known that key Na+ transporting proteins (amiloride-sensitive epithelial Na+ channels (ENaCs), Na+/K+-ATPases and Na+/H+-exchangers) contain numerous cysteine residues that are targets for intra- and extracellular oxidizing and reducing agents. However, after the addition of ENaC blocker amiloride (20 μM) to the medium bathing the skin apical surface, Na+ transport was completely inhibited. This indicates that the effect of glutoxim on Na+ transport is associated primarily with the modulation of ENaC activity.

Using a wide range of pharmacological agents affecting cellular signaling components we also found that in glutoxim regulation of Na+ transport in frog skin the tyrosine kinases, phosphatidylinositol kinases, protein kinase C, microtubules and microfilaments were involved. The results suggest that glutoxim may interact with the cysteine-rich domains of the insulin receptor in basolateral membrane of epithelial cells, induce its transactivation and trigger the signaling cascade, resulting in increased Na+ transport in frog skin.

**SW04.S16–267**

**Search for new antiviral compounds against human enteroviruses using fragment screening methodology**

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Picornaviridae are among the most diverse and oldest ‘known’ viral families that include many important pathogens of humans and animals. They are small,icosahedral ss+RNA viruses, causing a variety of diseases. Vaccines are available for PV, HAV and FMDV, but no effective prophylaxis is implemented for other picornaviruses. So far, anti-viral research has focused on the capsid, whereas inhibitors targeting non-structural proteins (i.e. proteases, helicases, polymerases) have remained largely unaddressed.

We are developing the project focused on searching for novel antiviral compounds against human enteroviruses (HEV) via fragment screening methodology based on STD-NMR technique. The protein target is picornaviridae protease.

Co-crystallization/soaking of the most successful STD hits with their protein target are being carried out to obtain their 3D structures by X-ray crystallography. The data provided by NMR and crystallography techniques will identify the close contacts between a fragment hit and a protein. It can help to infer the requirements underlying the association and suggest novel ligands by both fragment-growth and fragment-linking strategies. Consequently, new binders could be obtained that eventually will become leads for further development.

**SW04.S16–268**

**The most influential chemical structure features for rational drug design of prion disease therapeutics**

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Prion diseases are fatal neurodegenerative illnesses, due to accumulation of abnormal isoform of native prion proteins in the central nervous system. Although several studies have been done and many drug candidates proposed, there is currently no proven treatment or official drug for prion diseases. Even more, the molecular mechanism of post-translational conversion into misfolded isoform and cellular mechanisms induced by prion aggregations are still enigmatic. This study focuses on the antiprion activity prediction models, which could be effectively applied in further drug design of prion disease therapeutics. These models enable the categorization of compounds (active/inactive) and prediction of inhibition potency of new potential inhibitors obtained from virtual screening during the antiprion drug discovery process. The models developed and presented here are nonlinear, based on counter-propagation artificial neural networks (CP-ANN), and use molecular descriptors (MD) for input of chemical structures of compounds. For each novel compound of interest not solely in silico prediction, but also the evaluation of reliability and robustness of this prediction is given, as the models follow OECD principles by being externally validated and having defined the applicability domain. Moreover, with a technique of genetic algorithm the reduction of hundreds of molecular descriptors to the set of only ten of them was performed. The set of the most influential chemical structure features represented by selected molecular descriptors of antiprion compounds reveals a valuable guidance for rational drug design of prion disease therapeutics, because it gives information about properties of chemical structure considered for antiprion activity. Therefore, the selected variables having a potential impact on prion-prion interactions are interpreted and discussed.

**SW04.S16–269**

**Insulin superfamily peptides as a source of mutations in the design of new insulin drugs**

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Insulin superfamily is an ancient group of structurally homologous peptides, including insulin, IGF-I, IGF-II, relaxin, bombesin, and related peptides. Insulin-related peptides were identified in both vertebrates and invertebrates, including mammals, protochordates, nematodes, mollusks, and insects. Many of these hormones were shown to act via receptors structurally similar to the insulin receptor. Besides, both insulin and IGF-I can bind with a high affinity to their cognate receptors. This information was used for in the designing of pharmacologically effective insulin analogue, insulin LysPro, in which the native sequence at positions B28 and B29 is mutated to that of IGF-I to give the reverse sequence, leading to reduced formation of insulin dimers and hexamers. The aim of our study was to find other potential sites in the insulin molecule suitable for the development of new antidiabetic drugs using similarity in structures and conformational flexibility of the insulin superfamily peptides. We analyzed available primary, secondary, and tertiary structures of all insulin and insulin-like peptides. Based on the obtained data we selected several members of the insulin superfamily and assessed their conformational flexibility using molecular dynamics simulation.
method. Then we carried out docking of these peptides into the insulin receptor. Analysis of the conformational flexibility and ligand-receptor stability showed that A8 is the most favorable site for the introduction of mutations. We found that His and Lys mutants have greater intrinsic alpha-helical propensity than Thr and exhibit enhanced affinities and stabilities. Based on these data we can conclude that these peptides are the best candidates for the designing of new insulin analogues for treatment of diabetes mellitus.

**SW04.S16–270**  
**Pyrophosphate analogs suppress phosphorolytic activity of wild-type and AZT-resistant HIV-1 reverse transcriptase**  
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The first drug approved for the clinical treatment of HIV-1 infection was a nucleoside analog 3'-azido-3'-deoxythymidine (AZT). However, shortly after the introduction of AZT, cases of resistance were found. This resistance originates in the phosphorolytic excision of AZT by inorganic pyrophosphate or ATP catalyzed by viral reverse transcriptase (RT). In most cases, resistance originates from specific RT mutations called thymidine analog mutations (TAM). The mutations accelerate the excision of AZT so dramatically that AZT becomes almost ineffective.

Specific inhibitors of AZT-excision could have therapeutic utility, since they might restore the activity of the nucleoside based drugs against TAM-containing HIV. Optimally, such inhibitors should not reduce incorporation of AZT, since this would clearly antagonize anti-retroviral activity.

We present the results of an investigation of the inhibition of the PPI or ATP-mediated HIV-1 reverse transcriptase catalyzed phosphorolytic cleavage of AZT-terminated DNA primers by a series of 25 bisphosphonates (BPs). The influence of the structure of Mg²⁺ -coordinating group and an electron deficiency of the side-chain aromatic substituent of BPs on their activities were studied. We compared the ability of the most active BPs to suppress the PPI and ATP-induced excision catalyzed by WT or AZT resistant RTs (M41L, D67N, K70R, T215F, K219Q). For all BPs the correlation between their inhibiting activities in AZT-excision and DNA primer elongation processes were evaluated. We postulated that where is an almost linear dependence between the ability of studied BPs to inhibit of excision and elongation. Although it should be mentioned that BPs are more powerful inhibitors of excision then DNA elongation and have a minor influence on HIV RT catalyzed AZT-monophosphate incorporation into DNA.

**SW04.S16–271**  
**AZT prodrugs: achievements and trends in the treatment and prevention of HIV infection**  
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Despite the numerous drawbacks, 3'-azido-3'-deoxythymidine (AZT, Zidovudine, Retrovir) remains one of the key drugs used in the treatment and prevention of HIV infection in both mono-therapy and HAART. A strategy in searching for new effective and safe AZT agents among latent (depot) forms of AZT has yielded its positive results. In particular, AZT 5'-H-phosphonate (Nikavir) has demonstrated clinical advantages over parent AZT: first and foremost, lower toxicity and better tolerability. It can be effectively used for the prevention of vertical transmission from mothers to babies and as an alternative drug for HIV-infected patients with low intolerance to Zidovudine. Preclinical studies of another prodrug, AZT 5'-aminocarboxyphosphonate, have demonstrated that it releases AZT when taken orally. Pharmacokinetic studies have shown a prolonged action potential. Based on the analysis of both toxicological and pharmacological data, AZT 5'-aminocarboxyphosphonate has been recommended for clinical trials; preliminary results of clinical trials first stage are promising. O-(L-2',3'-Deoxyribose-3'-O-propyl)O-(3'-azido-3'-deoxythymidine-5'-yl)aminocarboxyphosphate can be regarded as a depot form of Zidovudine and Lamivudine, the drugs composing the anti-HIV cocktail Combivir. The activity of this compound exceeded those for parent AZT and 3TC both in vitro (in cell cultures infected by HIV-1) and ex vivo (human tonsil tissues infected by HIV-1). This phosphonate is stable in acid media and is rather rapidly hydrolyzed under neutral and alkaline conditions to give not only the parent nucleosides but also the corresponding 5'-aminocarboxyphosphonates, which are in turn depot forms of AZT and 3TC. The latest data for all the three AZT depot forms will be presented.

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**SW04.S16–272**  
**Methylenebysphophonates as new class of HIV-1 integrase inhibitors**  
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The integration of human immunodeficiency virus type 1 DNA into the infected cell genome is one of the crucial steps in the viral replication cycle. It is catalyzed by the viral integrase, which is thus a key potential target for new antiviral drugs. Only two integrase inhibitors, raltegravir and elvitegravir, are now approved for AIDS therapy. Unfortunately, treatment of patients with these drugs leads to the appearance of the resistant viral strains. The majority of integrase inhibitors currently at the stage of clinical trials are similar to raltegravir in terms of their mechanism of action. Raltegravir-induced cross-resistance to these compounds has already been demonstrated to develop in patients. Thus, designing new integration inhibitors that would differ from Raltegravir in terms of their mechanism of action is currently a pressing need.

We have found that some methylenebysphophonate derivatives (MBPs) inhibit integrase activity. SAR-analysis of these inhibitors allowed us identifying structural elements crucial for the inhibitory activity and understanding their mechanism of action. The inhibitory effect of MBPs is determined by the presence of chlorobenzyl substituent at the methylenebysphophonate backbone. All the active compounds inhibit both reactions catalyzed by IN, 3'-processing and strand transfer, with comparable efficiency. The mechanism of the IN inhibition by MBPs strongly depends on the nature of the second substituent at the methylenebysphophonate backbone; ω-amino-MBP is found to be non-competitive inhibitor whereas MBPs containing other substituents inhibit IN by competitive mode. Of note, all MBP inhibitors are active against integrases with mutations providing resistance to several known IN inhibitors including raltegravir.
These data lead us to conclude that these compounds show promise and need to be further studied as potential HIV-1 integrase inhibitors.

The work was supported by the Russian Foundation for the Basic Science (grants 12-04-33049, 11-04-01586).

**SW04.S16–273**

**Cytotoxic effect of human hepatitis A virus 3C protease is accompanied by cytoplasmic vacuolization**

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3C proteases take an essential role in picornaviruses’ lifecycle by processing viral polyproteins. Also, 3C proteases have several additional functions, some of them are caused by cleavage of host cell proteins (regulation of viral translation) and attenuation of host cell antiviral immunity and others are independent from proteolytic activity (recognition of viral RNA). Revealing new functions of 3C proteases is important for comprehension of virus-host cell interactions.

In this study we showed that human hepatitis A virus 3C protease (HAV3C) induced death of human lung cancer A549 and Calu-1 cells. Most of cells expressing HAV3C died within 72 hours, while catalytically inactive protease did not affect cells. With very minor exceptions, the dying cells did not show caspase activation and phosphatidyserine exposure. However, apoptosis-related events such as cytochrome c release, chromatin condensation and karyorrhexis were found almost for all dying cells. Inhibitors of caspases, z-VAD-fmk, lysosomal proteases, z-FAs-fmk and that of necroptosis, necrostatin-1 and ERK1/2 signalling pathway, PD98059 and Sc-353669 did not rescue the cells.

Substantial part of dying cells (up to 30%) demonstrated accumulation of cytoplasmic vacuoles, formed by lysosomes and several types of endosomes. Vacuolization was independent from Rab5 and Rab7 functions, which suggests non-specific fusion of the organelles. Vacuoles contained non-acidic media and accumulated extracellular liquid, while many acidic vesicles with active transporters were found almost for all dying cells. Inhibitors of caspases, z-VAD-fmk, lysosomal proteases, z-FAs-fmk and that of necroptosis, necrostatin-1 and ERK1/2 signalling pathway, PD98059 and Sc-353669 did not rescue the cells.

In conclusion, we showed that HAV3C induces caspase-independent cell death preceded by vacuolization of lysosomal-endosomal compartment, which resembles cytopathic phenotype produced by picornaviruses. The data suggest a role of HAV3C in development of cytopathic effect of human hepatitis A virus.

**SW04.S16–274**

**The WhiB7 gene polymorphism and its regulon genes in *Mycobacterium tuberculosis*, as a new mechanism of drug resistance**

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Almost 1/3 of the World’s populations are carriers of *M. tuberculosis* (*Mtb*), the main causative agent of tuberculosis (TB), in latent form. Thus *Mtb* is actually a constant component of much of humanity’s organisms and a risk factor for their health. TB treatment is complicated by the appearance of strains with multidrug- (MDR) and extensive drug-resistance (XDR). From 15% to 40% of mutations leading to MDR and XDR phenotypes are still not identified. WhiB7 is a key regulator of *Mtb* intrinsic drug resistance, and may play a role in MDR and XDR phenotypes. WhiB7 controls transcription of several genes (WhiB7 regulon), including 4, which play a role in drug resistance: Rv1258c (*tap*, aminoglycosides and tetracycline efflux pump), Rv1473 (macrolide-transport ATP-binding protein ABC transporter), Rv1988 (*erm*, 23S rRNA methyltransferases, conferring macrolide, lincomamide and streptogramin resistance) and Rv2416c (*eis*, enhanced intracellular survival protein Eis, GCN5-related N-acetyltransferase).

We’ve analyzed nucleotide sequences of *whiB7* gene and four genes of its regulon in full-length genomes of 63 *Mtb* strains stored on-line (http://www.ncbi.nlm.nih.gov/bioproject/12318) and found mutations in all of these genes. We’ve also analyzed by sequencing these five genes in 19 clinical isolates, 10 of which turned out to belong to Beijing phenotype. SNPs leading to amino acid sequence changes were found in these isolates. Among detected mutations was *whiB7ΔC1388*, a frame-shift mutation, leading to a shortened protein, missing a C-terminal domain, probably responsible for binding A/T rich DNA sites (A/T-hook), thus presumably non-functional.

W.t. and mutant *whiB7* genes were cloned in pET-32a vector and expressed in *E. coli*. They were also cloned in pMIND shuttle vector and transformed in *M. smegmatis* mc^2^ 155. *M. smegmatis* transformants were tested to tetracycline and macrolide antibiotics resistance.

**SW04.S16–275**

**Studies of RND-type efflux pump inhibitor Phenylalanylarginyl-beta-naphthylamide interaction with *Salmonella enterica* cells**

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Multidrug resistance efflux pumps are one of the major causes of antibiotic resistance in opportunistic pathogens such as *Salmonella enterica* ser. Typhimurium. Phenylalanylarginyl-b-naphthylamide (PAβN) is an universal inhibitor of resistance-nodulation-division (RND) family efflux pumps in Gram-negative bacteria. We studied efficiency of the efflux systems in *S. enterica* cells using method of microdilutions. PAβN is toxic to bacteria only at high concentrations but low inactive concentrations of ampicillin, chloramphenicol or tetracycline were preventing bacterial growth in combination with this efflux inhibitor. *S. enterica* cells with the permeabilized outer membrane (OM) were more sensitive to PAβN than the cells with intact OM. The efficiency of inhibitory activity of PAβN was dependent on the initial cell concentration used. Such results suggest intensive binding of this efflux pump inhibitor to cells. We used tetracyclines-phenolphthionium cations as the efflux pump substrate to monitor the efflux preventing efficiency of PAβN. Results of these measurements also indicated that the inhibitory efficiency of PAβN is higher in the case of *S. enterica* cells with the permeabilized OM. Finally, interaction of the inhibitor with bacteria was electrochemically monitored using PAβN–selective electrode. The measurements of PAβN binding indicated that EDTA-permeabilized cells accumulated considerably higher amounts of this inhibitor. This result explains the observed dependence of the inhibitory efficiency of PAβN on the permeability of the OM of *S. enterica* cells.
**SW04.S16–276**

**EU-OPENSSCREEN, chemical keys for life’s locks**

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Post-genome biology opens an immense scope of new targets for basic research and early drug discovery, but the availability of suitable ‘tools’ for systematic biochemical investigation of their function is lagging behind.

EU-OPENSSCREEN (www.eu-openscreen.eu) is an open access research infrastructure on the strategic European ESFRI roadmap (European Strategy Forum on Research Infrastructures) with the aim to develop novel research ‘tools’ (i.e. chemical inhibitors or activators of biological components) for all areas of the Life Sciences (incl. molecular, cell, plant, structural and microbiology; synthetic and medicinal chemistry; pharmacology and early drug discovery etc.).

External researchers are invited to apply for obtaining access to EU-OPENSSCREEN’s shared resources (i.e. cutting-edge screening technologies and equipment; unique compound collection; wide range of methods and assays; personal and expertise; training courses etc.) which support all stages of a tool development project.

The generated tool compounds, which will be made publically available, complement other genetic and molecular-biology methods and enable researchers to investigate the molecular mechanisms of physiological and pathological processes, many of which can only be properly studied with these chemical ‘tools’.

EU-OPENSSCREEN is expected to start operations in late 2015, but it can already look back on a growing number of transnational activities: creation of national interest groups, development of new design principles for its central compound collection; exchange of local compound libraries and joint screening projects.

**SW04.S16–277**

**Inhibitory effects of plant extracts and some chemical compounds on lipoygenase**

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Lipoxygenase (LOX, E.C. 1.13.11.12) activity has a significant detrimental effect on the organoleptic and nutritional quality of minimally processed fruits and vegetables. LOXs form a heterogeneous family of lipid peroxidizing enzymes being involved in the biosynthesis of inflammatory lipid mediators, such as leukotrienes, lipoxins, hepxolins, and other hydroxylated fatty acid derivatives. LOX is involved in provoking several inflammation-related diseases such as arthritis, asthma, cardiovascular, cancer, kidney and allergic diseases, neurodegenerative disorders and metabolic syndrome. For this reason, targeting inhibitors of LOX is a promising therapeutic target for treating wide spectrum of human diseases.

Plants still represent a large source of structurally novel compounds that might serve as leads for the development of novel drugs, nutraceuticals and functional foods. There is an increasing interest in naturally occurring antioxidants to replace synthetic counterparts used for food preservation, flavoring, and cosmetics, as well as in health promotion.

In this study, the inhibitory effects of 70% ethanol extracts and some chemical compounds prepared from different plants were investigated on the activity of LOX which has an important value in health area.

Ethanol extracts were prepared, filtered and evaporated to dryness in a rotary evaporator. All the extracts were dissolved in 70% ethanol before use. Chemical compounds were also dissolved in water. Inhibition of LOX with some plant extracts and chemical compounds were determined according to the methods of Yawer et al.

It was determined that all the plant extracts and chemical substances used in our study showed LOX inhibitory effect. The enzyme inhibitory activities of the extracts and chemical compounds were increasing in a dose-dependent manner. It can be suggested that plant extracts and chemical compounds which are potential sources of LOX inhibitors may be appropriate to be used as an additional support to drug treatment in the field of health.

**SW04.S16–278**

**Molecular genetic analysis of DNA-polymerases and thymidine kinases from clinical and laboratory HSV isolates resistant to ACV and HpACV**

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Human herpes simplex virus type 1 (HSV-1) is the most abundant pathogen accounting for herpetic diseases. When accompanying other diseases or in immunocompromised patients, HSV may cause serious health problems and even be lethal. Currently, HSV infection is treated mainly with acyclovir (ACV) and its analogs, which are phosphorylated in infected cells by several enzymes including viral thymidine kinases (TK), and the resulting triphosphates act as terminators of DNA synthesis. However, their prolonged use often leads to emergence of drug-resistant strains, which in most cases encode an inactivated viral TK gene. Therefore, search of new compounds effective against drug-resistant HSV strains is still important. Previously we described acyclovir 5’-H-phosphonate (HpACV), which exhibited activity against ACV-resistant TK- HSV-1 variants. Here we present an analysis of DNA-polymerase and TK genes from clinical and laboratory HSV isolates resistant to ACV and HpACV. Compared to the ACV-sensitive L2 strain, we identified several mutations in the DNA-polymerase gene: M880T, F716L, I529M, E545D, V585M, F716S, L1049M, N608S, N962D, A987T, F820Y, and G948S. Mutations were located in or close to the conserved domains and presumably affected DNA-polymerase activity and/or specificity, individually or in combination with other DNA-polymerase or TK mutations. Interestingly, some of these mutations were also observed in case of ACV-resistant HSV-1 strain. To explore mechanisms of formation of resistance to HpACV, we analyzed metabolism of the compound in uninfected Vero cells. It was revealed that Hp-ACV was not only hydrolyzed to give ACV but was also directly converted to the ACV 5’-monophosphate (ACVMP). Thus the compound could partially avoid phosphorylation by HSV-1 TK and retain activity against ACV-resistant TK strains. This work was supported by the RFBR (#12-04-00581-a) and RAS Presidium project ‘Molecular and Cellular Biology’.
SW04.S16–279
Molecular interactions with the bacterial cell wall by liquid state, standard and DNP solid state NMR
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The cell wall is essential for the survival of bacteria. It gives the bacterial cell its shape and protects it against osmotic pressure, while allowing cell growth and division. It is made up of peptidoglycan (PG), a biopolymer forming a multi-gigadalton bag-like structure, and additionally in Gram-positive bacteria, of covalently linked anionic polymers called wall teichoic acids (WTA). TAs are thought to play important roles in ion trafficking, host-cell adhesion, inflammation and immune activation.

The machinery involved in the synthesis of this envelop is crucial and is one of the main antibiotic target. Different protein as transpeptidase, transpeptidase activator or hydrolase are recruited to maintain the morphogenesis of the peptidoglycan during the bacterial cell cycle. Based on few examples involved in the machinery of synthesis of the peptidoglycan, we will demonstrate that a combination of liquid and solid-state NMR can be a powerful tool to screen for cell-wall interacting proteins in vitro and on cell.

In particular, structure of the L,D-transpeptidases that results in β-lactam resistance in M. tuberculosis, has been studied in presence of the bacterial cell wall and in presence of antibiotic. The NMR study reveals new insights into the inhibition mechanism.

In parallel, we have investigated the potential of Dynamic Nuclear Polarization (DNP) to investigate cell surface directly in intact cells. Our results show that increase in sensitivity can be obtained together with the possibility of enhancing specifically cell-wall signals. It opens new avenues for the use of DNP-enhanced solid-state NMR as an on-cell investigation tool.

References

SW04.S16–280
Insights into the inhibition of peptidoglycan L, D-transpeptidation
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The maintenance of bacterial cell shape and integrity is largely attributed to peptidoglycan, a biopolymer highly cross-linked through D,D-transpeptidation. Peptidoglycan cross-linking is catalyzed by Penicillin-Binding Proteins (PBPs) that are the essential target of β-lactam antibiotics. PBPs are functionally replaced by L,D-transpeptidases (Ldt) in ampicillin-resistant mutants of Enterococcus faecium and in wild-type Mycobacterium tuberculosis. Ldts are inhibited in vivo by a single class of β-lactams, the carbapenems, which act as a suicide substrate. We present here the first structure of a carbapenem-acylated L,D-transpeptidase, E. faecium Ldtfm acylated by ertapenem, which revealed key contacts between the carbapenem core and residues of the catalytic cavity of the enzyme. Significant reorganization of the antibiotic conformation occurs upon enzyme acylation. These results, together with the analysis of protein-to-carbapenem proton transfers, provide new insight into the mechanism of Ldt acylation by carbapenems.

References

SW04.S16–281
New nucleoside inhibitors of M. tuberculosis growth
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Recently, 5-modified pyrimidine nucleosides with lengthy 1-alkynyl substituents have demonstrated an inhibitory effect on M. tuberculosis and M. bovis in vitro [1–4]. The best compounds with antibacterial activity were 5-(1-dodecyl)- and 5-(1-tetradecyl)-derivatives. The SAR study of a carbohydrate fragment modification on antibacterial properties of 5-modified nucleosides has demonstrated that virtually all 2'-deoxy-, 2',3'-dideoxy-, 3'-fluoro-2',3'-dideoxy-, and 2'-fluoro-2',3'-dideoxynucleosides, as well as acyclic and arabinonucleosides with lengthy 1-alkynyl radicals, have displayed anti-TB activity [1–4]. This work is devoted to a synthesis and investigation of the M. tuberculosis growth inhibitory capability of the new series of 2'-deoxy-pyrimidine nucleosides containing lengthy alkoxymethyl radicals at their position 5 as well as various substituents at the carbohydrate moiety.

We have developed a method of pyrimidine nucleoside methoxyalkyl derivative synthesis [5] that is essentially easier and cheaper than the proposed in literature for 5-(1-alkynyl)-nucleosides [1–4]. We have studied the inhibitory effect of synthesized 2'-deoxynucleoside derivatives (Table 1) carrying a linear alkyl moiety introduced at position 5 of pyrimidine base via an oxymethyl group providing a higher flexibility of the hydrocarbon chain than 1-alkynyl derivatives described in literature [1–4] on the growth of M. tuberculosis. To reveal the role of 3'-modification of the carbohydrate moiety in the anti-TB activity of 5-modified nucleosides, we have synthesized the following 2'-deoxuridine derivatives with the same substituent at position 5 of the base: 3'-azido-2',3'-dideoxy- and 3'-amino-2',3'-dideoxy-5-dodecyloxyethyluridine. 5-Alkoxymethyl derivatives of pyrimidine nucleosides were tested in vitro at concentrations of 5, 10, 20, 40, 50, 100, and 200 μg/ml [6].
5-Dodecyloxy methyl-2-deoxyuridine and 5-dodecyloxymethyl-3'-amino-2-deoxyuridine can be regarded as a highly active inhibitors against M. tuberculosis (MIC<sub>100</sub> = 20 µg/ml), but the later is more toxic. The most important achievement of this work is that our nucleoside analogues demonstrated effective activity against multidrug resistant strain of M. tuberculosis (MIC<sub>100</sub> ≥ 20 µg/ml). Recently we synthesized a new series of such nucleosides carrying various substituents at 5'-position. Their antitubercular activity will be reported.

Thus, we have shown the capability of pyrimidine 2'-deoxynucleoside 5-methoxyalkyl derivatives to inhibit the growth of M. tuberculosis in vitro; the most effective derivatives could serve as prototypes for the development of new anti-TB drugs.

This study was partially supported by the Fundamental Research Program of the Presidium of the Russian Academy of Sciences ‘Molecular and Cell Biology’ and the Russian Foundation for Basic Research (grants №№ 11-04-00603 and 11-04-12035-ofi-m-2011).

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5. Alexandrova LA, Skoblov AY, Jasko MV, Victorova LS, O. A. Tsaplina
6. Expression of grimelysin or protealysin gene in S. proteamaculans is higher than that of S. grimesii 66. The expression of grimelysin or protealysin gene in S. proteamaculans is higher than that of S. grimesii 66. The complete genome sequence of S. proteamaculans 568 contains hemolytic toxin ShlA gene. Using PCR analysis with a set of primers specific for ShlA gene of S. marcescens this gene was detected in S. proteamaculans 94, but not in S. grimesii 66. The results of PCR analysis were confirmed by hemolytic activity of S. proteamaculans and the lack of the activity in S. grimesii 66. Moreover, iron limitation (induced by 2,2'-bipyridyl) increased both the hemolytic activity of S. proteamaculans and its invasion efficiency. S. grimesii cultured under the iron-restricted conditions did not exhibit any hemolytic activity and decreased its invasion efficiency.

To evaluate the effect of pore-forming toxin on invasion we used streptococcal hemolytic toxin streptolysin O as a model system. Incubation of HeLa cells with 5 mg/ml streptolysin O caused 1.5-fold increase of invasion by S. grimesii not producing ShlA and by recombinant E. coli expressing protealysin gene. However, this treatment did not modify susceptibility of HeLa cells to the non-invasive E. coli. These results indicate that the pore-forming hemolytic toxin may be involved in S. proteamaculans internalization but it is not indispensable for Serratia invasion.

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SW04.S16-283
Biotechnological process for the preparation of an antiviral drug ribavirin analogues substituted on the amide group
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Ribavirin (1-[β-D-ribofuranosyl-1,2,4-triazole-3-carboxamid, Virazole) is a modified nucleoside effective against a wide spectrum of RNA and DNA viruses. It demonstrates pronounced efficiency in treatment of hepatitis C and Lassa fever, as well as influenza of A and B types. Ribavirin, however, has serious side effects in the blood forming organs of patients: it causes hemolytic anemia. This fact motivates researchers to search for safer therapeutic drugs with less systemic toxicity. The chemo enzymatic (biotechnological) approach to the synthesis of ribavirin and its new analogues displaces the classic multistage chemical processes and allows carrying out the key reaction (transglycosilation) with high efficiency, region- and stereo-selectivity. Moreover, testing novel derivatives of 1,2,4-triazole as substrates of E. coli purine nucleoside phosphorylase (PNP) can reveal structural features of heterocyclic bases defining the potential for performing the reaction of the synthesis of modified nucleosides on their basis in the active site of the enzyme. This widens our view of the mechanism of the function and synthetic abilities of the enzyme. N-Cyclic aliphatic and aromatic 1,2,4-triazole carboxamides were found to be good substrates with the extent of conversion to the respective nucleosides up to 99%. Ten ribavirin analogues were prepared after optimization of the conditions of enzymatic reactions (the most effective ribose donor was identified, the nucleoside/base ratio and the optimal amount of enzyme were determined). A ribo- and 2-deoxyribo-nucleosides of N-substituted 1,2,4-triazole carboxamides were synthesized with a 30–90% yield. Unfortunately, this approach to the synthesis of nucleosides can not be used in the synthesis of arabinose- and 2-fluoroarabinose- nucleosides of 1,2,4-triazole. Investigation of antiviral activity of the synthesized compounds in the models of hepatitis C, herpes simplex virus type 2, type A and B flu viruses is currently being conducted in the Ivanovskii Institute of Virology. The test results of antiviral activity of the synthesized compounds will be published later.

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Serratia are facultative pathogens primarily causing nosocomial infections. The mechanisms of these infections are, however, poorly understood. Serratia marcescens is a most pathogenic species of this genus, and it has been the only Serratia species shown to possess invasiveness mediated by a pore-forming toxin ShlA (Hertle, Schwarz, 2004). We have previously found that S. grimesii 66 (Efremova et al., 2001; Bozhokina et al., 2008) and S. proteamaculans 94 (Tsaplina et al., 2009) are capable to invade eukaryotic cells provided that they synthesize intracellular metalloprotease grimesilin or protealysin, respectively. Therewith, invasion efficiency of S. proteamaculans 94 is higher than that of S. grimesii 66. Expression of grimesilin or protealysin gene in non-invasive E. coli confers an invasive phenotype (Bozhokina et al., 2011).

The complete genome sequence of S. proteamaculans 568 contains hemolytic toxin ShlA gene. Using PCR analysis with a set of primers specific for ShlA gene of S. marcescens this gene was detected in S. proteamaculans 94, but not in S. grimesii 66. The results of PCR analysis were confirmed by hemolytic activity of S. proteamaculans and the lack of the activity in S. grimesii 66. Moreover, iron limitation (induced by 2,2'-bipyridyl) increased both the hemolytic activity of S. proteamaculans and its invasion efficiency. S. grimesii cultured under the iron-restricted conditions did not exhibit any hemolytic activity and decreased its invasion efficiency. The most important achievement of this work is that our nucleoside analogues demonstrated effective activity against multidrug resistant strain of M. tuberculosis (MIC<sub>100</sub> ≥ 20 µg/ml). Recently we synthesized a new series of such nucleosides carrying various substituents at 5'-position. Their antitubercular activity will be reported.

Thus, we have shown the capability of pyrimidine 2'-deoxynucleoside 5-methoxyalkyl derivatives to inhibit the growth of M. tuberculosis in vitro; the most effective derivatives could serve as prototypes for the development of new anti-TB drugs.

This study was partially supported by the Fundamental Research Program of the Presidium of the Russian Academy of Sciences ‘Molecular and Cell Biology’ and the Russian Foundation for Basic Research (grants №№ 11-04-00603 and 11-04-12035-ofi-m-2011).
SW04.S16–284
Disubstituted uracils as novel nonnucleoside inhibitors of HIV-1 reverse transcriptase inhibitors
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Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are important components of a Highly Active Antiretroviral Therapy (HAART). They bind to a specific hydrophobic pocket in the structure of reverse transcriptase (RT) adjacent to the polymerase active site of the enzyme. Because of a high flexibility of NNRTI binding pocket, various types of compounds were reported to inhibit HIV RT. One of the most promising group of NNRTIs is comprised of diarylpyrimidine (DAPY) derivatives, with two of them, namely etravirine and rilpivirine, approved by FDA for treatment. However, search for new NNRTIs of this type remains an important goal for modern medicinal chemistry.

Recently we reported two groups of N1,N3-disubstituted uracil derivatives as potent HIV RT inhibitors. Despite that fact that most of the compounds of the first group (oxyethyl derivatives) showed moderate activity, the most active compound exhibited submicromolar activity towards HIV RT (K0.5 = 0.26 μM). Another group of compounds comprised of the cinnamyldervatives, displayed higher activity, but were less soluble. The most promising inhibitors of these two groups were evaluated towards a panel of mutant RTs corresponding to the drug-resistant HIV strains. It revealed that N1,N3-disubstituted uracils retained pronounced activity against the L100I and the G190A mutants and partially retained against the K103N, V106A and Y181C mutants. However, HIV RT bearing double mutation K103N/ Y181C was resistant towards these types of compounds. Therefore, future attempts for structure optimization are needed to improve the inhibition activity and to develop the compounds with the desired biological properties.

SW04.S16–285
Role of myeloperoxidase binding with the surface of low-density lipoproteins in their proatherogenic modification by reactive halogenated species
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One of the factors promoting oxidative/halogenating modification of low-density lipoproteins (LDL) is myeloperoxidase (MPO). Earlier we have shown that MPO binds to the LDL surfaces. The LDL–MPO complex is uncoupled in the presence of peptide EIQQDCTGDDED, which imitates a fragment of apob-100 (445–456). In this paper we studied how this peptide, as well as inhibitors and modulators of halogenating activity of MPO – ceruloplasmin (CP), 4-aminobenzoic acid hydrazide (ABAH) and thiocyanate (SCN-) – affect the accumulation of cholesterol and its esters by monocytes/macrophages after incubation with LDL subjected to different kinds of MPO-dependent oxidative/halogenating modification. It is shown that MPO in the presence of H2O2 and halides leads to higher proatherogenic modification of LDL than exogenic reactive halogen species (HOCI and HOBr).

Both monocytes, which differentiate into macrophages, and neutrophils secrete MPO in response to the presence of damaged LDL. The peptide, which prevents the MPO–LDL interaction, reduces the entrapment of modified LDL and MPO by monocytes/macrophages, preventing the accumulation of intracellular cholesterol. The results indicate the importance of MPO binding to the LDL surface for modification of LDL and acquisition of their proatherogenic properties. The EQIQDCTGDDED peptide, CP, ABAH, and SCN- can play the role of anti-atherogenic factors, which reduce the damaging effect of catalytically active MPO on LDL and the accumulation of cholesterol by macrophages.

SW04.S16–286
Inclusion of antituberculous drug rifampicin into phospholipid-oleate nanoparticles as a way for efficiency increase
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The formulations of phospholipid nanoparticles <30 nm with embedding of a number of lipophilic drugs was elaborated by us earlier and increase of drugs bioavailability and efficiency was shown. The goal of this work was the search of corresponding nanosystem for antituberculous drug rifampicin that was induced by increased tuberculosis incidence in last decades. Opposite to a number of other drugs, rifampicin poor embedded into nanoparticles composed of only phosphatidylcholine, but the adding of sodium oleate was found to increase drug loading more than twofold. The nanosystem based on soy phosphatidylcholine and sodium oleate with particle size <30 nm was developed with use of high pressure homogenization. The formulation of rifampicin included into nanoparticles is prepared in the freeze-dried form suitable for storage. Particle size and degree of drug incorporation didn’t change after hydration. Rifampicin in this formulation showed increased antituberculosis efficiency in mice infected with M. tuberculosis Erdman. After 6 weeks of oral administration the value CFU (colony forming units) in lung was in 22 times smaller than for free drug (1.7 versus 37.4). One of the reason of such effect is suggested to be the increase of drug bioavailability – it was far more higher (11.3 times) for rifampicin in phospholipid-oleate nanoparticles as compared with free drug administration. Besides, rifampicin in nanoparticles revealed in vitro more active inhibition of cell M. tuberculosis H37Rv: growth: minimal inhibiting concentration was twice smaller than for free rifampicin (0.5 versus 1 mg/ml). It suggests the more drug penetration into bacterial cells, possible due to affinity of both nanocarriers excipients – phospholipids and oleate – for high hydrophobic M. tuberculosis cell wall. Thus, the elaborated phospholipid-oleate nanoparticles appeared as the new drug delivery nanosystem for rifampicin that significantly increases drug efficiency. It allows to decrease drug doses and side effects, and therefore to improve the quality and results of tuberculosis therapy.
SW04.S16–287
HCV core protein induces oxidative stress and activates antioxidant defense system by several distinct mechanisms
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Hepatitis C virus (HCV) is a human pathogen which causes chronic liver disease often accompanied by extraparitie complications. Patients with chronic hepatitis C often develop liver fibrosis, cirrhosis, and hepatocellular carcinoma, as well as steatosis, iron overload and insulin resistance. These events are triggered by the oxidative stress induced by HCV in infected cells. The most potent stress inducer is the nucleocapsid (core) protein, which activates production of reactive oxygen species (ROS) by in mitochondria, or by activating NADPH: oxidases (NOX1, NOX4). Previously we showed that core protein also activates antioxidant defense Nrf2/ARE pathway by ROS-dependent and Nrf2/ARE-independent mechanisms. We investigated mechanisms of induction of oxidative stress and Nrf2/ARE activation in Huh7 cells expressing HCV core. We showed that the protein stimulated ROS production by several independent mechanisms. (i) The N-terminal core domain (aa 1–36) activates expression of NOX1 and NOX4 transforming growth factor (TGF-β)-dependent fashion. (ii) C-terminal domain (aa 37–191) activates expression of cytochrome P450 2E1 (CYP2E1). (iii) The same domain induces ER oxidoreductin–1 (aa 1–191) core forms.

In addition, we showed that C-terminal domain of HCV core was responsible for induction of Nrf2/ARE defense pathway via ROS-dependent activation of protein kinase C. In the same time, ROS-independent activation of Nrf2 was observed in case of N-terminal fragment of the core protein. These data demonstrated that these two mechanisms were independent and were not mediated via any unknown upstream mediator.

SW04.S16–288
Antimalarial activity of cupredoxins: the interaction of Plasmodium merozoite surface protein 19 (MSP119) and rusticyanin
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Finding effective new antimalarial agents is an urgent need. One of the most studied molecules anchored on the parasite surface is the C-terminal fragment of the Merozoite Surface Protein 1 (MSP119), which is taken into the red blood cell at invasion and remains intact in the digestive food vacuole to the end of the intracellular cycle. Thus, MSP119 is a promising target against malaria since a number of specific antibodies inhibit erythrocyte invasion and parasite growth. Given the structural homology of cupredoxins family with Fab domain of monoclonal antibodies and previous evidences of parasitemia inhibition due to the interaction of azurin with MSP119, an approach combining NMR and ITC measurements with Biacore-based docking calculations is performed on MSP119/cupredoxins complexes. Among tested cupredoxins, rusticyanin forms a well-defined complex with MSP119 in a site that overlaps with the surface recognized by the inhibitory antibodies. These findings become rusticyanin as an excellent therapeutic tool for malaria treatment and provide valuable information in drug design.

SW04.S16–289
Antibodies against ectromelia virus capable of neutralizing variola virus: generation and application for epitope mapping.
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Virus-neutralizing antibodies are very important in host defense against viral infection. One possible approach is the selection therapeutic antibodies against orthopoxviruses, naturally occurring in immunized donors using phage display methods. Development of antibody against variola virus and monkeypox virus is accompanied by necessity work in BSL 3-4 biosafety level, which leaves many limitations and is resource-intensive. The use of non-pathogenic virus in the first stages selection of recombinant antibodies would significantly simplify, reduce the cost, and, possibly, speed up the creation of therapeutic antibodies. It is known that the surface proteins of orthopoxviruses highly homologous, and in response to infection with one type of animal models various orthopoxviruses work enough similar pattern of specific antibodies. Given this, we have used in the non-pathogenic to humans CE5/35 for selection of phage antibodies against the variola virus. Five unique pdAbs against ECTV was isolated from a combinatorial phage display library of human scFv antibodies constructed from the Vh and Vi genes cloned from the peripheral blood lymphocytes of vaccine-immune donor. The specificity was confirmed by ELISA, AFM and Western blot analyses. VH domains of selected anti-ECTV antibodies belong to the VH3-9 subfamily human immunoglobulins and had a considerable resemblance to each other. Three of 5 unique to the virus ectromelia selected phage antibodies were able to inhibit the infectivity of variola virus on a monolayer of eukaryotic cells. One pdAb displayed a dose-dependent neutralization for all the tested viruses VARV, MPXV, VACV, and CPXV. Thus, we confirmed the possibility of selecting anti-ECTV antibodies when used as an antigen for biopanning and screening of non-pathogenic to humans ectromelia virus.

Target protein for neutralizing pdAbs shown to bind a protein with a molecular mass of approximately 35 kDa, corresponding to the main immunodominant protein in the development of orthopoxvirus infections in humans. This protein is encoded by an open frame broadcast H3L nomenclature vaccinia virus strain Copenhagen. Profile-based Kyte-Doolittle hydrophobicity index and antigenicity Jameson-Wolf, the likely epitopes were predicted protein p55. According to the probable epitopes and predicted
binding site for heparan sulfate in order to localize the site responsible for the interaction with neutralizing antibodies was established panel deletion variants of the protein p35. For the production of recombinant plasmid DNA, providing a shorter version of the protein expression of p35 in the cells of E. coli, was used as a vector plasmid pUR291, containing the gene encoding β-galactosidase. The ability of the selected phage antibodies against ectromelia virus link shortened version of the p35 protein was investigated by Western blot analysis. The results showed that the antibody reacted with two shorter versions, and do not reveal the rest. Thus, in the interaction with neutralizing antibodies involved amino acids located in the region corresponding to 130–257.

SW04.S16–290
Design and study of artificial HIV-1 polyepitope immunogens optimized for inducing HIV-specific immune responses
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For intracellular pathogens, such as HIV-1, the conventional naïve T cells reactive with short peptide epitopes presented by major histocompatibility complex (MHC) class I and II proteins on the surface of an infected cell are critical for protective immunity. Therefore artificial HIV-1 polyepitope immunogens based on selected HIV-1 T-cell epitopes realize a promising approach for inducing effective CD8+ HIV-specific immune responses.

Three artificial immunogens TCI-N1, TCI-N2 and TCI-N3 have been designed on the basic knowledge about antigen processing and presentation. Using original software TEpredict and PolyCTLDesigner 50 CD8+ and 5 CD4+ T-cell epitopes in the structure of HIV-1 antigens have been predicted with the conservation of 80% among A, B and C HIV-1 isolates. Designed immunogens contain optimized core polyepitope sequence polyE and three flanking ‘signal’ sequences in different combinations, including N-terminal ubiquitin, N-terminal signal peptide and C-terminal tyrosine motif of LAMP-1 protein to improve efficacy of different strategies of polyE processing and presentation on the pathways of MHC-I and MHC-II restriction. For enhancement of CD8+ and CD4+ T-cell responses highly conservative HLA-DR binding T-helper peptides and ‘PADRE’ peptide were included in the structure of target immunogens. Genes encoding designed immunogens were cloned in vector plasmid pCDNA3.1 and three DNA vaccine constructs have been obtained: pCDNA3.1_Kozak_polyE (TCI-N), pCDNA3.1_Kozak_ER_signal_polyE_LAMP-1 (TCI-N2) and pCDNA3.1_Kozak_Ub-V70_polyE (TCI-N3). Target genes expression in vitro after transfection of 293-T cells with obtained plasmids have been evaluated with mRNA detecting, Western blot analysis and with flow cytometry using antibodies to p24 marker epitope. Immunogenicity of engineered vaccines has been studied on mice model after DNA immunization with created plasmids. Intracellular cytokine staining assay was applied to evaluate HIV-specific CD4+ and CD8+ responses in this study.

We showed that all plasmid constructs provide intracellular processing and gene expression of engineered artificial immunogens. Following immunization all investigated DNA vaccines elicited HIV-specific cellular immune responses. Immunogens contained ubiquitin and LAMP-1+ER-signal show tendency for increasing CD8+ HIV-specific responses.

SW04.S16–291
Determination of CYP2C9 gene polymorphisms in myocardial infarction patients
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The incidence and prevalence of acute myocardial infarction (MI) increase progressively and it’s the major cause of morbidity and mortality in the world. Cytochromes P450 (CYP) 2C8 and 2C9 are partly responsible for the biosynthesis of vasoregulating endogenous substances e.g. the arachidonic acid metabolites epoxyeicosatrienoic acids (EETs), also known as endothelium-derived hyperpolarizing factors (EDHFs). Which CYP2C9 product, that is, EETs or oxygen-derived free radicals, is most relevant for the regulation of vascular tone in individuals with coronary artery disease is not yet clear. We investigated whether the polymorphisms of CYP2C9 is related with myocardial infarction. The study population consisted of 48 MI patient and 63 unrelated healthy individuals. Blood was collected in EDTA-containing tubes and DNA was extracted from leukocytes by High Pure PCR template preparation kit. CYP2C9*2, CYP2C9*3 allele were detected by using a LightCycler-CYP2C9 mutation detection kit in real time PCR.

We found that distributions of CYP2C9*2 wild and heterozygous genotypes in the MI patients were 68.3%, 31.7% and in the controls were 87.3%, 12.7%, respectively. CYP2C9*3 wild and heterozygous genotypes in the MI patients were 64.6%, 35.4% and 85.7%, 14.3% in controls, respectively. CYP2C9*2 heterozygous genotype was associated with a 3.1-fold (OR = 3.1; 95% CI = 1.19–8.16, p = 0.02) and CYP2C9*3 heterozygous genotype was associated with a 3.2-fold (OR = 3.2; 95% CI = 1.31–8.26, p = 0.01) increased risk of MI. In conclusion, CYP2C9*2 and CYP2C9*3 heterozygous genotypes may have a critical role in MI.

SW04.S16–292
The role of lectin-like oxidized LDL receptor-1 as a mediator of endothelial dysfunction in patients with metabolic syndrome
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Background and aim: Metabolic syndrome (MetS) defines a well-known cluster of metabolic disturbances associated with an increased risk of cardiovascular disease and diabetes. In this study in patients with MetS and healthy controls the levels of soluble lectin-like oxidized LDL receptor-1 (sLOX-1), it’s possible association with oxidized LDL (oxLDL), endothelial nitric oxide synthase (eNOS), nitric oxide (NO), endothelin-1 (ET-1), paraoxonase 1 (PON1) and arylesterase (ARE) activities were examined.

Methods: Fifty-five patients (37 women, 18 men) with MetS and 29 healthy controls (19 women, 10 men) with a body mass index (BMI) less than 25 kg/m2 were enrolled in the study. sLOX-1, oxLDL, eNOS and ET-1 were determined by ELISA; NO, PON1 and ARE were measured by colorimetry.

Results: When compared to healthy controls the levels of sLOX-1, oxLDL, and ET-1 were significantly higher (p = 0.023, p < 0.001, and p < 0.001, respectively) but the levels of eNOS,
NO, PON1 and ARE were significantly lower (p = 0.017, p < 0.004, p < 0.001, and p = 0.010, respectively) in patients with MetS. sLOX-1 was observed to be positively correlated with ox-LDL, ET-1, BMI, glucose. ET-1 also exhibited significant negative correlation with ARE activity.

**Conclusions:** We have shown that in patients with MetS high sLOX-1 levels are associated with cardiovascular risk factors such as increased oxLDL, obesity and increased fasting blood glucose. An increased concentration of LOX-1 could be considered as an early predictor of endothelial damage in MetS. In addition, it appears that oxLDL, ET-1, eNOS, NO, PON1 and ARE may accurately reflect the levels of endothelial dysfunction in MetS patients.

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**SW04.S16–293**

The investigation of lipoprotein associated phospholipase A2 (Lp-PLA2) V279F single point mutation in coronary artery disease

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The main lesion of coronary artery disease (CAD) in the early phase of atherogenesis, chronic inflammatory response to oxidative modification of LDL is thought to lead to the subendothelial accumulation of inflammatory cells, such as macrophages and T lymphocytes. Lp-PLA2 (lipoprotein associated phospholipase A2) is an enzyme that belongs to the A2 phospholipases family that hydrolyzes phospholipids at the sn2 position generating potent proinflammatory and proatherogenic products, such as lysophosphatidylcholine and oxidized free fatty acids from oxidation of LDL. Various mutations especially V279F mutation observed in Lp-PLA2. Because of V279F mutation reducing the affinity of Lp-PLA2 substrate, the aim of this study was to determine the possible role of Lp-PLA2 V279F polymorphism in patients with CAD. In this study, 180 subjects who underwent coronary angiography by Mersin University Medical Faculty Department of Cardiology were included. 109 subjects who have ≥70% stenosis in any of the major coronary arteries were selected as CAD and 71 subjects who have no stenosis or lesion were selected as control. DNA of control and CAD groups were extracted from whole blood. Lp-PLA2 V279F polymorphism was detected by using Lp-PLA2 V279F mutation detection kit with real-time PCR method. For Lp-PLA2 V279F mutation, VV (wild) genotype was detected in both the CAD group and the control group while VF (heterozygous) and FF (mutant) genotypes were not detected. In conclusion, because of not detected heterozygous and mutant genotypes in our study, the Lp-PLA2 V279F mutation can not be evaluated as a genetic risk factor.

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**SW04.S16–294**

Combating bacterial antibiotic resistance: Novel lactamase inhibitors

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The treatment of bacterial infection using beta-lactam antibiotics, including penicillin and cephalosporin compounds, is currently limited by bacterial resistance to these agents, typically caused by beta-lactamase enzymes. More than 800 mutations have been identified in enzymes of this type. Existing inhibitors of beta-lactamases can prevent antibiotic degradation arising from beta-lactam ring hydrolysis, but due to the additional development of resistance to these compounds, they are not sufficiently effective. Thus, the identification of new inhibitors and new mechanisms of lactamase inhibition is a priority.

To address this challenge, we computationally screened 8 million organic molecules using the ViCi software (http://www.embl-hamburg.de/vici), which we specially developed for this purpose. The software permits the rapid screening against a known inhibitor template and selects the closest matching compounds in terms of shape and electrostatic composition. Four known low-affinity lactamase inhibitors were given to the software as a starting point. Recombinant TEM-1 enzyme was produced and used to assay 550 of the top compounds suggested by ViCi screening. Five new inhibitors were identified, the best having an order of magnitude higher in vivo affinity for the enzyme than its template. Intriguingly, two of these are predicted to be allosteric inhibitors and three others are based on inhibitors of CTX-M-9, suggesting that cross-inhibition of different isoforms may be possible. The inhibition was confirmed in vivo.

X-ray crystal structure determination of the structures of native enzyme and its complexes with ligands is now in progress. The ViCi software is being improved to accept multiple templates and will be used to suggest compounds for the next iteration of screening. Docking protocols, validated by crystallography, may be used to refine the results, aiding our rapid convergence to more avidly binding compounds. The current status of the X-ray crystallographic, computational and laboratory studies will be presented.

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**SW04.S16–295**

Discovery of the new antiviral drug among nitrusubstituted azolo-azines

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During the long term study, the original molecule 2-methylthio-6-nitro-1,2,4-triazolo[5,1-c]1,2,4-triazin-7(4H)-one (Triazavirin), was identified as a compound a with broad spectrum of antiviral activity. Triazavirin (TZV) as substance was identified as a moderate replication inhibitor of influenza virus A and B, including A(H5N1) virus, and tick born encephalitis. The activity of TZV has been proven in NIIAD against West Nile hemorrhagic fever.

During the analysis of mechanism of action of TZV it was established that there are two reactive groups are contributing to the antiviral activity – S-methyl at the 2nd position and NO2-group at the 6th position of triazolo-triazine heterocycle.

According to the test results, the condition eliciting the highest level of antiviral activity is the presence of the NO2-group. NO2-group is activated when the living cells are treated with the free radicals. The hypothetical molecular mode of action is based on free radical activation of NO2-group of TZV molecule and reac-
tion of peroxynitrite radicals (NOO – ONOO⁻) with proteins of the thiol-group. Such modification is similar to protein nitrosylation and leads to damage of viral proteins. Taking into account the induction of iNOS in all stages of influenza infection and hyperproduction of NO and ONOO⁻, there is a strong dependence of TZV action even in the late period of complicated disease. TZV passed through preclinical studies and complete clinical trials phases I-III. Also there is some evidence concerning interfering of this modification with protein disulfate isomerase cellular enzyme which is important for maintenance of viral infectivity.

**SW04.S16–296**

**The role of lipid metabolism and the formation of antibodies to neuronal proteins in the development of diabetes mellitus type 1**

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It is widely known that different types of stress induce prolonged increase in free fatty acids (FFA) content in human blood, which results in inflammation. This may lead to the blood-brain barrier damage and to the exit of neuronal proteins into the bloodstream and the activation of synthesis of antibodies to these proteins. We suppose that these antibodies interaction with neuronal proteins from endocrine cells may result in loss functional activity of endocrine cells. In the case of beta-cells this may lead to decrease in insulin secretion and increase in blood glucose levels.

To test this suggestion we measured FFA content in the blood serum of children with diabetes mellitus type 1 (DM1) by gas chromatography. We observed about two times increase in FFA content in blood of diseased children.

Our studies included patients with DM1 who have no clinical signs of distal neuropathy. With the help of electromyography it was found that 70% of ill children displayed loss of peripheral nerve conductivity. 30% of these patients were characterized by increased level of antibodies to neuronal proteins, whereas in the control group only 6% children had increased content of such antibodies. We suppose that the change in functional activity of nerve cells may be determined by the increased content of this type of antibodies which may affect activity of cells of other types.

To this end, mice were immunized twice by mice brain homogenate. It was observed that after the second immunization by measurement of blood glucose content some mice had increased glucose level (8–10 mmol/l) for 7–10 days.

These results demonstrate simultaneity in development of two illnesses – diabetes mellitus type 1 and neuropathy and allow to suggest that DM1 induction may be caused by increase in FFA content and suppression of CNS and PNS functional activity.

**SW04.S16–297**

**Role SNPs of CD40 in predisposition to multiple sclerosis**

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The interactions of CD40 and CD40L have been known to critically regulate B-cell responses. Thus, CD40 is a likely candidate to play roles in autoimmune diseases such multiple sclerosis (MS) in which activated T and B cells cause pathology. In 2009, Australia and New Zealand Multiple Sclerosis Genetics Consortium was identified risk-associated SNP upstream 6 kb of CD40 on chromosome 20q13 (rs6074022, P = 1.3 x 10⁻⁶). [1]. An association between rs6074022 and MS was also identified in a GWAS based on a cohort from the United Kingdom [2]. However, in the meta-analysis reported in 2011, the association between SNP rs6074022 and MS did not reach the GWAS significance level (p = 4.91 x 10⁻⁵) [2]. Latter, Blanco-Kelly F et al. identified association between rs1883832, a polymorphism located at -1 from the start codon of the CD40 gene, and MS [3].

The aim of this work was to study the association between these two and other two CD40 SNPs and multiple sclerosis from Russian Federation. Moreover we would like to estimate location causative SNP.

Determination of genotypes of CD40 SNPs (rs6074022, rs1883832, rs1535045, rs11086996) was performed by Real-time PCR. Statistical analysis was performed using the R-language.

In this work, we studied the association between four CD40 SNPs and multiple sclerosis in 1679 cases and 879 controls from Russian Federation. Two SNPs were associated with MS: rs6074022 (OR = 1.27, 95%CI [-1.12-1.45] p=0.0003, per allele) and rs1883832 (OR = 1.20, 95%CI [-1.05-1.38] p=0.007, per allele), whereas rs1535045 and rs11086996 were not.

These two associated SNPs are in strong linkage disequilibrium (D'=0.82, r=0.59) and mapped within a promoter region of the gene. Maximum likelihood analysis showed that the model, including rs6074022, is significantly better (p=0.01) than model, including rs6074022 and rs1883832. Summarizing the data, we may speculate that the functional variant(s) is likely to be located in the upstream region of the gene CD40 and is in higher LD with rs6074022 than with rs1883832.

**References**


**SW04.S16–298**

**Killing bacteria from the inside: genetically encoded Trojan horses to give insight in bacterial cell death**

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The 2008 Chemistry Nobel Prize recognized the discovery, expression and development of the green fluorescent protein. Since its finding, novel fluorescent proteins (FPs) have been engineered to tailor its properties [1]. The development of mutants able to generate reactive oxygen species (ROS) is pursued as a tool in microscopy, cromophore assisted light inactivation or photodynamic therapy [2]. Our study focus on two promising mutants in terms of ROS production.

We first demonstrated that TagRFP is capable of photosensitizing the production of singlet oxygen (O₂). When expressed in
E. coli bacterial cell death could be induced in a light-dose dependent manner. This demonstrate that intracellular generation of $^{1}O_2$ is sufficient to kill bacteria, which paves the way for the development of novel approaches to overcome antibiotic resistance [3].

Recently, we have turned our attention to miniSOG, an efficient ROS-producing flavoprotein [4]. We have demonstrated its potential as genetically encoded photosensitizer photoinactivating bacteria more efficiently than any other FP. We are currently studying the scope and mechanistic details of its antimicrobial activity [5].

References

SW04.S17 Biochemistry of Neoplastic Transformations (IV-S17)

SW04.S17–1
Therapeutic efficacy of B cell receptor signaling inhibitors in lymphoma

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The Staudt laboratory developed genomic-scale gene expression profiling to define the molecular basis of therapeutic response and survival in lymphoid malignancies. This effort revealed that the most common type of non-Hodgkin’s lymphoma, diffuse large B-cell lymphoma (DLBCL), is actually comprised of three pathogenically distinct diseases with different responses to current chemotherapy. To uncover new therapeutic targets, the Staudt laboratory developed RNA interference genetic screens for essential genes in cancer. Using this methodology, in conjunction with high throughput cancer gene resequencing, the laboratory identified the NF-κB, B cell receptor and MYD88 signaling pathways as therapeutic targets for the ABC subtypes of DLBCL, which is least curable by current treatments. Gain-of-function mutations in the B cell receptor components CD79B and CD79A occur in more than one-fifth of ABC DLBCL tumors, demonstrating the pathogenetic importance of the B cell receptor pathway in this lymphoma subtype. Clinical trials are underway in relapsed/refractory DLBCL of ibrutinib, a small molecule inhibitor of the kinase BTK in the B cell receptor signaling pathway. A high frequency of complete and partial responses to ibrutinib have been observed in ABC DLBCL, consistent with the reliance of this lymphoma subtype on chronic active B cell receptor signaling. By contrast, the response rate in the GCB DLBCL subtype, which does not generally rely on B cell receptor signaling, is low. Responses have been seen in primary refractory tumors that were resistant to all prior chemotherapies. Several of these heavily pre-treated patients have survived more than 1 year and one has been disease-free for 2 years while taking single agent ibrutinib without discernible side effects. Characterization of recurrent genomic aberrations and their relationship to ibrutinib response will be discussed, but notably, mutations in the B cell receptor signaling pathway are not required for complete and partial clinical responses. New mechanistic insights into the genetics of chronic active B cell receptor signaling in ABC DLBCL will be presented.

SW04.S17–2
Understanding activation mechanisms of growth factor receptor tyrosine kinases

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The 58 human receptor tyrosine kinases (RTKs) fall into 20 families that are characterized by distinct combinations of domain types in their extracellular ligand-binding regions and the nature of their intracellular tyrosine kinase domains. The different RTK families are activated through distinct mechanisms, and (where relevant) characterized by different sets of activating oncogenic mutations. I will discuss our recent investigations of RTK signaling mechanisms in four receptor families, drawing parallels and distinctions as relevant. First, I will summarize our studies of the epidermal growth factor receptor (EGFR), generally viewed as a ‘prototypic’ RTK, and the target of several important cancer therapeutics. I will describe unique allosteric regulation mechanisms seen in EGFR and other ErbB family members, which provide an explanation (through negative cooperativity) for the ‘high-affinity’ and ‘low-affinity’ binding sites for EGF seen at the cell surface. I will also outline our progress in studies of the EGFR relative ErbB3, which contains a so-called ‘pseudokinase’ in its intracellular region. Other important RTK families with intracellular pseudokinases include the Ryk and Ror families, thought to be regulated by extracellular binding of Wnt ligands. I will describe our recent progress in understanding how Wnts can regulate these RTK families, combining structural and functional studies of both the extracellular regions (and their binding to Wnts) and the unusual intracellular kinase domains. Finally, I will describe how our understanding of the tyrosine kinase domain activation mechanism in another RTK, called ALK (anaplastic lymphoma kinase), can aid patient treatment decisions in neuroblastoma.

SW04.S17–3
In search for pan-cancer promoter

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The application of gene therapy in oncology often exploits the transcriptional targeting – an approach allowing restriction of the therapeutic gene expression to target tumor cells. The cellular gene regulatory elements active in desirable tumors are usually used for this purpose. The regulatory system should provide sufficient specificity and expression of the transgene. Usually, natural tissue- or tumor-specific promoters are used for this goal. However, all known cancer-specific promoters are relatively weak as compared with the strong constitutive CMV or SV40 promoters. In addition, most of the promoters described are active only in a limited number of cancer cell types. A perfect universal cancer-specific (pan cancer) promoter should be active in wide
A delivery system that could efficiently reach tumor, engage receptors on the cancer cell surface and transport its payload to the cell nucleus would be attractive for maximizing both the specificity and effectiveness of tumor treatment. We created modular nanotransporters (MNT) that are polypeptides containing at least two functions: (i) an ability to direct nanotransporter combination PhTERT-PhSurv269 with a shortened PhTERT promoter; it revealed the highest and more uniform promoter expression level in different cancer cells in comparison with all other cancer specific promoters tested. The tandem also retained cancer-specificity. In other approach, we evaluated the cancer specificity of variety promoters involved in control of cell proliferation. All tested promoters were found to be significantly more active in cancer cells than in normal fibroblasts, thus providing new cancer specific regulatory elements for the transcriptional targeting. The results will be discussed from point of view (i) of the mechanisms of the transcriptional activities of the promoter investigated and (ii) from point of view of their application for gene therapeutic purposes.

**SW04.S17-4**

**Modular nanotransporters: a versatile platform for nuclear delivery of anti-cancer pharmaceuticals**

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A delivery system that could efficiently reach tumor, engage receptors on the cancer cell surface and transport its payload to the cell nucleus would be attractive for maximizing both the specificity and effectiveness of tumor treatment. We created modular nanotransporters (MNT) that are polypeptides containing at least four moieties: (i) an internalizable ligand to provide target cell recognition and receptor mediated endocytosis by the cell, (ii) an endosomolytic module to facilitate escape from endosomes, (iii) a nuclear localization sequence to allow active transport into the cell nucleus, and (iv) a carrier domain. MNT can increase in vitro cytotoxicity of photosensitizers and α-particle emitters up to a factor of 3,000. MNT selectively accumulate in vivo in cancer cells, with the highest concentration in cell nuclei. Importantly, MNT mediated delivery of photosensitizers resulted in more than 90% longer tumor growth delay and significantly prolonged survival compared with free drug, while producing few if any side effects. Moreover, MNT can deliver a wide spectrum of substances; they possess low toxicity and low immunogenicity in mice; their production and purification is simple and cost effective; it is possible to replace MNT modules to exploit different molecular and subcellular targets and to freeze-dry MNT for prolonged storage.

**SW04.S17-5**

**Lysozyme dependent apoptosis induced in Ehrlich ascites carcinoma cells**

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It is well known that carbohydrate profile of plasma membranes of normal and malignant cells is strongly different. In particular plasma membranes of many malignant cells are sialated. Moreover the preferential binding of wheat germ agglutinin (N-acetyl-glucosamine binding lectin) to malignant cells supposes that plasma membranes of malignant cells are enriched by these sugars too. It is supposed that these carbohydrates masked some receptors and as a result these changes in carbohydrate profile can determine growth features of these cells. So a question arises whether the changes in the carbohydrate profile of plasma membranes alter malignancy of cells and whether this process can have therapeutic significance. We have applied lysozyme as the N-acetyl glucosamine destroying enzyme for testing of this possibility. Effect of the enzyme on Ehrlich ascite carcinoma (EAC) was studied. It was shown that lysozyme in concentration of 0.1 and 0.01 mg/ml brings to apoptosis of these cells as was judged from DNA-comet and DNA fragmentation. Parallel to this, an increase in the level of lactate dehydrogenase which is the indicator of necrosis is observed. In contrast lysozyme in same concentration has no such effects on normal leukocytes. So pro-apoptotic effect of lysozyme on EAC cells is demonstrated. The potential role of endogenous macrophage lysozyme in monitoring of malignization processes is discussed.

**SW04.S17-6**

**Methylation of PTEN gene promoter and PTENP1 pseudogene in endometrial and ovarian tumors**

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Genetic mutations in tumor suppressor gene PTEN are often found in human malignant cells and such genome changes are especially characteristic of endometrial cancer. In our previous researches we assumed that an alternative epigenetic mechanism of PTEN gene inactivation through promoter methylation may exist in endometrial cancer. Moreover, PTENP1 pseudogene has recently been shown to play a role in positive regulation of PTEN gene expression. The 3′ untranslated region (3′ UTR) of PTENP1 mRNA competes with the 3′ UTR of PTEN mRNA for regulator micro RNAs that inhibit PTEN expression at the level of translation through RNA interference. Therefore PTENP1 pseudogene transcription inhibition in tumors through aberrant methylation may be a new mechanism of PTEN expression suppression. In this connection, the aim of our study was to investigate the methylation status of PTEN gene core promoter and the 5′ region of PTENP1 pseudogene in endometrial cancer, endometrial hyperplasia and ovarian cancer. Tumor tissue samples were obtained from 46 patients of the N.N. Blokhin Cancer Research Center (Moscow). Normal endometrial tissue samples from 13 healthy women and peripheral venous blood samples from 10 healthy donors were also used in the study. Genomic DNA was isolated and purified by standard methods. Methyla-
tion status of PTEN and PTENP1 was analyzed using various approaches: methyl-sensitive sequencing, COBRA and methyl-sensitive PCR (in the case of PTENP1 pseudogene analysis). It was discovered that PTEN gene promoter was not methylated in any of the tumor DNA samples analyzed by us. Nevertheless, the 5' region of PTENP1 pseudogene was methylated in 12 of the 19 endometrial cancer samples, in seven of the 13 endometrial hyperplasia samples and in three of the 14 ovarian cancer samples. Only one of the 13 normal endometrial tissue DNA samples had PTENP1 pseudogene methylated. PTENP1 methylation was not found in any of the 10 DNA samples obtained from peripheral venous blood of healthy donors. Our hypothesis is that PTENP1 methylation may suppress PTEN transcription, in accordance with the competitive endogenous mRNA theory, and may play a role in endometrial and ovarian cancers pathogenesis.

SW04.S17–7
Alpha-fetoprotein: 50-year anniversary as cancer biomarker
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Discovery of phenomenon of biosynthesis of AFP, the major mammalian embryo-specific antigen, during carcinogenesis by Garry Abelev and Yuri Tatarinov 50 years ago, in 1963, provoked intensive studies of this protein. Attempts of different scientific groups throughout the world resulted in isolation, purification and physico-chemical and immunological characterization of AFPs from some mammalian species. Despite the significant success in study of AFP, its three-dimensional (3D) structure has not been experimentally elucidated yet. Also, mechanisms of receptor binding along with structure of the receptor itself and gene that encodes its biosynthesis remain still obscure. Over the last decade, identification of functionally important sites by comparison of primary structures of AFP and some physiologically active proteins with the use of computer-based sequence alignment has been undertaken. This has led to prediction of functions of AFP and to suggestions of multi-modularity and poly-functionality of this protein. Structural–functional mapping of AFP has been performed. Some peptide fragments of AFP have been synthesized and tested for biological activity. Among them are growth inhibitory peptide (GIP) and its fragments, EGF-like heptapeptide LDSYQCT (AFP14-20) and its fragment analogs, immunodominant sites CAKENAVE (residues 193–200) and GVALQTMKQ (residues 542–550), and others. To elucidate molecular mechanisms underlying biological activity of some of these peptides molecular dynamics (MD) simulation study was carried out and roles of certain amino acid residues and intramolecular interactions were revealed. Modeling of 3D structure of AFP-DES complex was performed to study mechanisms of interaction between AFP and the hormone. Besides, over the last years, detection of circulating AFP mRNA level also has been proposed to be used in diagnosis and prognosis of hepatocellular carcinoma. Moreover, interfering micro-RNA to suppress AFP gene and to inhibit proliferation of HCC cells and tumor growth along with usage of AFP as a vector for targeted delivery of anti-tumor drugs also have been proposed.

SW04.S17–8
Cancer associated fibroblasts and M2 polarized macrophages synergize during prostate carcinoma progression
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Inflammation is now acknowledged as an hallmark of cancer. Cancer associated fibroblasts (CAFs) force a malignant crosstalk with cancer cells culminating in their epithelial mesenchymal transition (EMT) and achievement of stemness traits. Herein we demonstrate that stromal tumor-associated cells cooperate to favor malignancy of prostate carcinoma. Indeed, prostate CAFs are active players of monocyte recruitment towards tumor cells, mainly acting through stromal derived growth factor-1 (SDF-1) delivery, and promote their trans-differentiation towards the M2 macrophage phenotype. The relationship between M2 macrophages and CAFs is reciprocal, as M2 macrophages are able to affect mesenchymal mesenchymal transition of fibroblasts, leading to their enhanced reactivity. On the other side, prostate carcinoma cells themselves participate in this crosstalk through secretion of monocoyte chemotactic protein-1 (MCP-1), facilitating monocyte recruitment and again macrophage differentiation and M2 polarization. Finally, this complex interplay among cancer cells, CAFs and M2-macrophages, cooperates in increasing tumor cell motility, ultimately fostering cancer cells escaping from primary tumor and metastatic spread, as well as in activation of endothelial cells and their bone-marrow-derived precursors to drive de novo angiogenesis. In keeping with our data obtained in vitro, the analysis of patients affected by prostate cancers at different clinical stages, revealed a clear increase in the M2/M1 ratio in correlation with clinical values. These data, coupled with the role of CAFs in carcinoma malignancy to elicit expression of stem-like traits, should focus great interest for innovative strategies aimed at the co-targeting of inflammatory cells and fibroblasts to improve therapeutic efficacy.

SW04.S17–9
Implication of alpha-5/beta-1 integrin in invasion of human breast carcinoma cells: a role for MMP-2 collagenase
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Although integrin α5β1 is one of the most studied integrins in cancer, its functions in different aspects of this disease have not been completely elucidated. In particular, controversial data exist on its role in tumor invasion and metastasis. In order to establish mechanisms underlying involvement of α5β1 integrin in invasion, we depleted its expression in MCF-7Dox human breast carcinoma cells via siRNA. We demonstrated that concomitant to α5β1 integrin depletion, was a sharp decrease in MMP-2 collagenase expression and inhibition of the invasiveness of these cells in vitro. Similar reduction of invasion potential was observed upon siRNA-mediated silencing of the MMP-2 gene. Downregulation of α5β1 integrin was accompanied by a substantial decrease in the amounts of active (phosphorylated) forms of Akt, Erk1/2

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kinases and c-Jun oncoprotein. Moreover, in MCF-7Dox cells, blocking the activity of above kinases by specific inhibitors strongly reduced expression of MMP-2 and c-Jun, and suppressed invasion of the cells in vitro. Similar results were observed upon siRNA-mediated silencing of c-Jun expression. Co-immunoprecipitation experiments demonstrated that Zβ1 integrin interacts with MMP-2 collagenase on the surface of MCF-7Dox breast carcinoma and SKMel-147 human melanoma cells. Our data suggest that Zβ1 integrin controls invasion of the studied cells via regulation of MMP-2 collagenase expression which can occur either through signaling pathways involving PI-3K, Akt and Erk protein kinases and the c-Jun or via direct recruitment of MMP-2 to the cell surface.

**SW04.S17–10**

**Characterization of oncogenic properties of gene encoding chitinase 3-like 1 protein (CHI3L1)**

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Identification of those genes whose copy number and expression increase during tumor development is important for understanding of oncogenesis. CHI3L1 encoding the secreted chitinase 3-like 1 protein was among genes with the most pronounced increased expression in gliomas. Significant increasing of CHI3L1 expression was shown also in a number of other malignant tumors. Given that CHI3L1 is overexpressed in many human cancer cells, we addressed the question whether CHI3L1 possesses transforming properties. It was demonstrated that cells stably produced CHI3L1 protein (293_CHI3L1) had the decreased doubling time and anchorage-independent growth in soft agar that is the most consistent indicator of oncogenic transformation. ERK1/2 and AKT are activated in the 293_CHI3L1 cells. Stable CHI3L1 expression made 293 cells tumorigenic: these cells stimulated the initiation of tumors after their xenograft transplantation into the brains of adult immunocompetent rats. In vivo MRI study revealed tumors in seven of 10 rats with implanted 293_CHI3L1 on the 21st day after implantation. Huge tumors with approximately similar volume were visible also both in allogenic (C6 glioma) and other xenogenic model (U251), while no tumor growth was observed after implantation of primary human lung adenocarcinoma cells, 293 cells, and 293-pcDNA3.1 cells. Tumors contained the dense superficial cell layer and prominent lobules with central newly ingrowing blood vessels that may signal angiogenesis which is a major process in carcinogenesis. Positive results with transplantation of 293_CHI3L1 cells into adult rat brains without any immunosuppression proved the validity of proposed animal model that displays the possibility to study various biological features of and host therapeutic response to brain tumor in an immunocompetent host. To elucidate the relationship between CHI3L1 and tumorigenesis, we employed a CHI3L1 gene knock-down approach by siRNA transfection in 293 cells, which stably produced CHI3L1. Blockade of CHI3L1 expression in 293 cells, which stably produced CHI3L1, decreased the ability of these cells to grow in soft agar and activity of MAPK signaling cascade. Thus the overexpression of CHI3L1 is likely to be critical in the development of some tumors and it could be used as one of the potential targets for complex anticancer treatment. Heterotransplantation of human brain tumor cells or tumor biopsies into immunocompetent rodents can be used as an alternative preclinical approach for the development of novel cancer therapeutics.

**SW04.S17–11**

**PDK1 regulates epithelial cell migration through MRCK**

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3-Phosphoinositide Dependent Kinase 1 (PDK1) is an AGC kinase able to bind and phosphorylate other protein kinases belonging to the AGC family. A lot of evidence has suggested the central role of PDK1 in cell migration but the molecular mechanisms regulating this process have yet to be understood.

According to our results, PDK1 regulates the directional migration of MCF10A mammary cells towards Epidermal Growth Factor (EGF). PDK1-downregulation leads to a marked reduction in the chemotactic capacity of MCF10A cells, while its over-expression increases their migration capacity. Significantly, this process does not involve the kinase activity of PDK1. Instead it depends on binding to PIP3 at the membrane and on its PIF binding pocket which mediates the interaction with its downstream effectors. We also found evidence that PDK1 regulates Myosin Light Chain 2 (MLC2) phosphorylation. Among the kinases able to phosphorylate MLC2 we identified Myotonic Dystrophy kinase-related Cdc42-binding Kinase z (MRCKz) as a direct interactor of PDK1. MRCKz and PDK1 colocalize at the lamellipodia membrane upon EGF stimulation and regulated lamellipodia dynamic. Therefore, the effects of PDK1 overexpression on cell migration were hampered by MRCKz downregulation or inhibition.

In summary, these findings not only identify a novel signaling pathway activated by PDK1 but also support the role of MRCKz in epithelial cell migration.

**SW04.S17–12**

**The acute cytotoxicity and lethal concentration (LC₅₀) of Agaricus sylvaticus mushroom through hemolytic activity on human erythrocyte**

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There is limited information regarding acute toxicity and lethal concentration of edible and medicinal mushrooms. The objective of this paper is to estimate the cytotoxicity of the aqueous extract of *Agaricus sylvaticus* mushroom on human erythrocytes by determining the lethal average concentration (LC₅₀). Six concentrations of the mushroom (17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/ml) were submitted for evaluation of hemolytic activity in vitro, using a suspension of blood. Through the Prism GraphPad Software, using the Tukey test for statistical analysis (p ≤ 0.05), a curve was constructed with values of *A. sylvaticus* mushroom concentrations versus the values determined by absorbance spectrophotometry at 540 nm. Results of hemolytic activity for the aqueous extract were fitted using nonlinear regression and the equation: \( Y = a x/(b + x) \). We used values of y as hemolytic activity and x as log of *A. sylvaticus* mushroom concentration.

The coefficient for determining the curve \( R^2 \) was 0.95 of the original data. The percentage of haemolysis increased in a concentration-dependent manner of *A. sylvaticus* extract used. The LC₅₀ value obtained was 9.213 mg/ml. Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.
SW04.S17–13
Cytotoxicity of A. sylvaticus mushroom in non-tumor cells (NIH/3T3) and tumor (OSCC-3) using tetrazolium (MTT) assay
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The purpose of this study was to assess the cytotoxic effect of the non-fractionated aqueous extract of A. sylvaticus mushroom in cultures of non-tumor cells (NIH3T3) and tumor cells (OSCC-3). The cells were maintained in DMEM cell culture medium added of 10% of fetal bovine serum and 1% antibiotic. For the cytotoxicity test we prepared the aqueous mushroom extract at concentrations of 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 mg/ml. From the values of the optical densities we determined the drug concentration analysis was performed using 570 nm wavelength. From the values of the optical densities we determined the drug concentration analysis was performed using 570 nm wavelength. The IC50 of 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 mg/ml. For the culture test we prepared the aqueous mushroom extract at concentrations of 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 mg/ml. Therefore, the mushroom has no cytotoxic effect of the non-fractionated aqueous extract of A. sylvaticus mushroom in cultures of non-tumor cells (NIH3T3) and tumor cells (OSCC-3). The cells were maintained in DMEM cell culture medium added of 10% of fetal bovine serum and 1% antibiotic. For the cytotoxicity test we prepared the aqueous mushroom extract at concentrations of 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 mg/ml. From the values of the optical densities we determined the drug concentration analysis was performed using 570 nm wavelength. From the values of the optical densities we determined the drug concentration analysis was performed using 570 nm wavelength.

SW04.S17–14
Novel molecular mechanism of Akt-dependent down-regulation of Pdcd4 tumor suppressor in lung cancer cells
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Pdcd4 is a tumor suppressor which is frequently down-regulated in various tumors thus making Pdcd4 an attractive target for anti-neoplastic therapy. Down-regulation of Pdcd4 expression contributes to neoplastic transformation, tumor development and progression. Pdcd4 also suppresses invasion and intravasation – two initial steps of metastasic cascade. Suppression of Pdcd4 occurs due to transcriptional silencing, miR21 microRNA-dependent transcript degradation and inhibition of translation, and also because of proteasomal ubiquitin-dependent degradation of Pdcd4 protein directed by PI3K/Akt/mTOR-signaling pathway. We have confirmed that P13K/Akt/mTOR-signaling indeed significantly contributes to Pdcd4 suppression in lung cancer cells but not only due to promoting Pdcd4 protein degradation. Our data show that inhibition of Akt-signaling pathway leads to up-regulation of Pdcd4 transcript. Using reporter gene system we showed that Akt-signaling pathway in an mTOR-dependent manner directly suppresses transcriptional activity of Pdcd4 gene promoter, while repressing Akt/mTOR signaling axis activity cancelled this suppression. Deletion or mutation of putative transcription factor binding site in the Pdcd4 promoter abrogated effect of Akt pathway inhibition on induction of transcription thus pinpointing regulatory element responsible for this phenomenon. Taken together, we described a novel mechanism of Akt-dependent suppression of Pdcd4 in tumor cells consisting from mTOR-dependent inhibition of Pdcd4 gene transcription, which might contribute to pro-oncogenic activity of Akt signaling pathway. These findings also contribute to understanding of complex molecular mechanisms responsible for Pdcd4 suppression in cancer in particular, and for tumor development and progression in general.

SW04.S17–15
GHRH-mediated transactivation of EGFR in human androgen-independent prostate cancer cells
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Growth hormone-releasing hormone (GHRH) and epidermal growth factor receptor (EGFR) are promoters of cell proliferation, migration and adhesion in prostate cancer. The aim of this work was to study the cross-talk between the signaling pathways in which both type of receptors are involved. Therefore, we analyzed the in vitro effect of GHRH on the activation of EGFR and several elements implicated in such effect as well as the in vivo outcome of the GHRH antagonist, JMR-132. For this purpose, a human androgen-independent prostate PC3 cancer cell line was used. Firstly, we observed that phosphorylated EGFR (p-EGFR) levels were enhanced after cell incubation with GHRH (0.1 μM) with the highest expression at 30 s and 30 min. The response to GHRH was mediated by specific binding of the neuropeptide to GHRH receptors since cell pre-incubation with JMR-132 (0.1 μM) completely blocked GHRH-induced EGFR tyrosine phosphorylation. Furthermore, protein kinase inhibitors (H89 for PKA and PP2 for Src kinase) and specific inhibitors of metalloproteinases (GM6001 for MMPs and TAPI-1 for ADAMs) were able to block GHRH-mediated effects at 30 s and 30 min on p-EGFR, respectively. Secondly, we analyzed the in vivo effect of JMR-132 in a xenograft mouse model. For this purpose, cells were injected into nude mice in order to induce PC3 tumors. Semiquantitative RT-PCR together with Western blotting showed decreased expression levels of both EGFR mRNA and protein, after treatment with JMR-132 (10 μg/day). The results shed light on the mechanisms of action of GHRH and the inhibitory effect of its antagonist in androgen-independent prostate cancer. These findings support the merit of further studies on the potential usefulness of GHRH receptor antagonists and EGFR antibodies or tyrosine kinase inhibitors for androgen-independent prostate cancer therapy.

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SW04.S17–16
Relation of eNOS/NOS3 genotypes and oxidative stress markers in larynx cancer patients
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Nitric oxide is a short-lived small molecule that has key functions in signal transduction process. Despite to its wide biofunctions, role of NO in oxidative stress mechanism seems to be critical. Endothelial nitric oxide synthase (eNOS or NOS3) is the responsible enzyme from production of NO in various cell types. The G894T variation of NOS3 was assumed with functional importance and causes an amino acid substitution at which results production of an enzyme variant with aspartic acid at position 298, instead of glutamic acid. This was reported to alter the susceptibility to cleavage of NOS3 thus, decreased endothelial NO production. This specific variation has been associated with several diseases including various cancer types.

In current study we have examined the possible relationship between NOS3 G894T genotypes and various oxidative damage markers such as superoxide dismutase (SOD), total thiol (T-SH), non-protein thiol (NP-SH), protein thiol groups (P-SH) and lipid hydroperoxides (LHP) in patients of larynx cancer.

29 larynx cancer patients and their 30 respective controls were included to study. Genotyping was carried out by PCR-RFLP method. Oxidative damage parameters were determined with colorimetric methods. In larynx cancer patients with TT genotype, SOD activities and NPSH levels were significantly higher than the controls (p = 0.021 and p = 0.022 respectively). In patients with GT genotype, high levels of LHP showed high significance (p < 0.001) when compared to controls. Within the patients groups, patients with TT genotype showed significantly increase levels of NP-SH when compared to GT and GG genotypes (p = 0.02).

These results indicate a potential relationship between G894 T variation of NOS and increased levels of oxidative stress markers thus represents a possible risk and progression parameter for larynx cancer patients. However, further studies are needed to clarify the molecular mechanisms of our findings.

SW04.S17–17
p53 and NFKB in CXCR5 gene regulation in human breast carcinoma cells
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Elevated expression of chemokine receptors in tumors is associated with abnormal activation of prosurvival intracellular pathways and increase of tumor cell metastatic potential via promotion of tissue-specific infiltration. The objective of this work was to assess the intracellular pathways involved in chemokine receptor CXCR5 gene expression in human breast cancer cells. We focused on p53, the key tumor suppressor, and transcription factors of NFκB family that often play an important prooncogenic role.

P53 expression in MCF-7 breast cancer cells was suppressed by lentiviral transduction of p53-directed shRNA. The role of NFKB in CXCR5 transcription was studied by bioinformatical analysis and directed mutagenesis of CXCR5 promoter in pGL3 luciferase reporter system. P53 suppression resulted in a significant increase in (i) CXCR5 promoter activity, (ii) CXCR5 mRNA and protein levels, and (iii) CXCL13-dependent migration of MCF-7 cells. Since no p53-binding sites were found in CXCR5 promoter region, we suppose that p53 effect on CXCR5 promoter activity should be indirect. Mutation of NFκB-binding sites resulted in a strong decrease of CXCR5 promoter activity, so NFKB appears to be one of the key mediators of CXCR5 gene expression activity.

Based on our data we can conclude that p53 could regulate CXCR5 expression level via repression of NFκB activity or by inhibiting some other stages of NFKB-dependent CXCR5 gene activation.

SW04.S17–18
Involvement of p53 in the antitumoral effect of VIP in human clear cell renal cell carcinoma
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Introduction: The molecular mechanisms involved in the development and progression of clear cell renal cell carcinomas (ccRCCs) are poorly understood. We previously showed in these cells that VIP is able to reduce cell proliferation and VEGF levels and to increase ROS production through phosphoinositide3-kinase (PI3K) signaling. Moreover, VIP is involved in other antitumoral events since it reduces cells migration and invasion, as well as the expression of survival signals (STAT3 or NFKB) (Vacas et al., 2012, 2013). The present study was undertaken to investigate p53 involvement in the antitumoral effect of VIP in human VHL-null A498 ccRCC cells.

Methods: Cell adhesion and invasion assays, ELISA of VEGF165 and ROS production assay were performed. RT-PCR and Western blot were used to evaluate p53 expression. The role of p53 in VIP effect was investigated with p53 inhibitor Cycloheximide (CPTF-aH).

Results: VIP (1 μM) up-regulated p53 levels in a time-dependent manner and this increase was implicated in the effect of VIP on cell adhesion, ROS production and VEGF165 secretion, since it was abolished by the p53 inhibitor. Furthermore, the increase of p53 produced by VIP was abolished by the P13K inhibitor wortmannin.

Conclusion: These results allow us to implicate p53 in the VIP antitumoral effects through activation of P13K. Moreover, the role of VIP in preventing invasion and metastasis in A498 cells support the potential therapeutic value of VIP in ccRCCs.

Keywords: VIP; P13K; VEGF; ROS; p53; Renal cell carcinoma.
Patients with premalignant lesions of cervix show increased lipid peroxidation and changes in antioxidative system, what may suggest presence of oxidative stress in the early phase of carcinogenesis. Paraoxonase 1 (PON1) is a calcium-dependent esterase, synthesized in the liver and secreted into the plasma where is associated with HDL. PON1 hydrolyzes different substrates by its organophosphatase, arylesterase and lactonase activities. Also, PON1 possess antioxidant/antiatherogenic activity, and it removes radicals formed by lipid peroxidation in human body.

We carried out this study in order to determine PON1 paraoxonase activity in patients with premalignant lesions of the cervix.

Study included fifty-three women [38 (19–38) years] with diagnosis of cervical intraepithelial neoplasia (CIN) that was confirmed by biopsy. 17 patients were classified in CIN1, 17 in CIN2 and 18 in CIN3. Eighty three healthy volunteers [37 (29–43) years], without any cervical or other disease, were enrolled in the study, as a control group. PON1 paraoxonase activity was assayed spectrophotometrically with paraoxon as a substrate in absence and in presence of NaCl. We standardized PON1 basal and salt stimulated paraoxonase activity in relation to the concentration of HDL-cholesterol. Standard lipid parameters, concentration of triglyceride, total cholesterol, HDL cholesterol, LDL cholesterol were determined by commercially available reagents.

Serum concentrations of determined lipid parameters were similar between two study groups. However, concentration of HDL cholesterol was significantly reduced in CIN patients [1.5 (1.3–1.6) mM versus 1.8 (1.6–2.1) mM; p < 0.001]. PON1 basal activity [104 (66–226) U/L versus 89 (67–193) U/L; p = 0.344] as well as salt-stimulated paraoxonase activity [210 (131–397) versus 179 (132–346) U/L; p = 0.345] were similar between two study groups. There was no statistically significant difference in PON1 activities standardized to the concentration of HDL cholesterol (basal paraoxonase activity 52 (39–130) versus 62 (44–129) U/mmol; p = 0.291; salt-stimulated paraoxonase activity 102 (75–235) versus 121 (86–230) U/mmol; p = 0.300).

Our results show that PON1 paraoxonase activity is not reduced in patients with CIN. Although these patients have reduced HDL concentration that reduction does not result with changes in PON1 activity. However, it is important to point out the fact that PON1 activity was determined with synthetic substrate (paraoxon) and that activity do not necessarily correlate with antioxidiant ability of PON1.
SW04.S17–22
Omega-3 PUFA modulate p-glicoprotein (Pgp) activity altering lipid raft cholesterol
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P-glycoprotein (Pgp) is a member of the ATP-binding cassette transporter superfamily, which confers efflux of its substrates from the cytosol to extracellular space. Pgp overexpression determines multidrug resistance (MDR), decreasing sensitivity of cancer cells to a wide variety of cytotoxic compounds, as doxorubicin, thus influencing therapy efficacy. Pgp is a membrane pump that interacts with the lipid surrounding environment, and it is mainly localized in lipid rafts. In particular, membrane cholesterol is essential to maintain a high Pgp activity [1].

Several studies have demonstrated that ω-3 polyunsaturated fatty acids (ω-3 PUFA) intake is correlated to low incidence of colorectal cancer [2]. The mechanism(s) by which ω-3 PUFA might exhibit a protective effect remains unclear but one of the hypotheses indicates that they may also change the fluidity of cell membrane and thus influence signaling pathways.

We studied the effects of ω-3 and ω-6 PUFA (EPA, DHA and AA) in human doxorubicin-sensitive (HT29) and doxorubicin-resistant (HT29-dx, overexpressing Pgp) colon cancer cells.

EPA and DHA are incorporated in plasma membrane, especially in lipid rafts, altering lipid composition. After treatment with 50 µM PUFA, cholesterol content is decreased in whole cell membranes and also lipid rafts of HT29-dx.

HT29-dx have also higher cholesterol synthesis compared to sensitive cells. EPA and DHA treatment induces a decrease of cholesterol synthesis starting from 50 µM in HT29-dx. Moreover, ω-3 PUFA, especially DHA, determine a reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) activity and expression in HT29-dx, increasing ubiquitinated enzyme and probably its degradation.

ω-3 PUFA do not modify Pgp content in whole cells, but reduce its localization in lipid rafts. Consistently DHA and EPA reduce the Pgp activity, measured as the ability to extrude rhodamine and induce doxorubicin accumulation and cytotoxicity in drug resistant HT29-dx cells, measured as decreased cell viability and increased necrosis.

In conclusion ω-3 PUFA reduce Pgp activity and chemosensitize colon MDR cancer cells to the Pgp substrate doxorubicin, by altering the microenvironment of lipid rafts.

References

SW04.S17–23
Study on the expression pattern of histone demethylases in HPV-induced cervical lesions
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Introduction: Epidemiologic data confirmed high risk human papillomaviruses (hrHPV) as etiological agents of cervical cancer but other cofactors are considered to be important in cervical carcinogenesis. Recently, histone lysine-specific demethylases (KDMs) have been shown to contribute to the development and progression of many types of cancer (acting either as tumour suppressors or oncogenes, depending on their gene regulatory function).

Objective: In order to investigate the possible role of KDMs in cervix oncogenesis we analysed their expression pattern in patients with HPV-induced cervical lesions and cancer.

Material and Methods: Cervical specimens from women with hrHPV-induced dysplastic cervical lesions (LSIL/HSIL) (n = 64, age range: 22–63 years, median: 36) and tissue specimens from patients with squamous cervical carcinoma (SCC) (n = 22, age range: 22–69 years, median: 36) were investigated. All samples were tested for hrHPV DNA presence (Linear Array, Roche). 30 women (age range: 20–53 years, median: 35) with negative cytological smears and HPV DNA negative were included as control group. Total RNA isolated from the samples (using Trizol reagent) was used to generate cDNA. KDMs expression levels were quantified in Real-Time PCR (Taqman). Statistical analysis was performed using Graph Pad Prism and Kruskal-Wallis test was used.

Results: Our study showed a differential KDM pattern of expression in cases versus controls. A strong correlation between hrHPV-positive precancerous (LSIL/HSIL) lesions and KDMs expression levels was noticed (p < 0.05). We found a significant gene overexpression for KDM1B (mean n-fold = 1.912; p = 0.015), KDM2B (mean n-fold = 0.809; p = 0.0079), KDM4C (mean n-fold = 1.621; p = 0.028) and KDM5C (mean n-fold = 1.492; p = 0.057 in LSIL/HSIL lesions, while KDM3C (mean n-fold = 1.495; p = 0.035) and KDM6A (mean n-fold = –3.65; p = 0.046) and KDM6B (mean n-fold = –3.58; p = 0.0061) presented a considerably reduced expression. Furthermore in all SCC subjects, an increased risk of HPV infection was also associated with the expression of the selected KDMs (p < 0.02).

Conclusions: These findings suggest KDMs display a differential expression pattern in HPV-induced cervical lesions and cancer, making them possible candidates as tumour suppressor or oncogenes in cervical carcinogenesis but their role in the mechanism of tumour initiation and progression remains to be solved.

SW04.S17–24
Role of ZNF224 in drug-induced apoptosis in chronic myelogenous leukemia
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The protein ZNF224 is a member of the KRAB-zinc finger family of transcriptional repressor. Recently, we demonstrated that ZNF224 is a novel human WT1-associating protein and that ZNF224/WT1 interaction modulates the expression of apoptosis regulating genes [1, 2]. Moreover, we observed that cytokine arabinoside (ara-C) induces ZNF224 expression in chronic myeloid leukemia (CML) cell lines and that ZNF224 overexpression enhances the apoptotic effect mediated by ara-C in K562 cells [2].

In the present study, we show that CML cell lines, positive for BCR/ABL translocation, express significantly lower amounts of ZNF224 respect to BCR/ABL negative cell lines. Moreover, we demonstrate that transfection of a BCR/ABL expression vector in KG1 cell line down-regulates ZNF224 mRNA and protein expression, thus indicating that ZNF224 is negatively modulated by this aberrant kinase activity. Finally, we observe that ZNF224 expression is induced by Imatinib, a Bcr-Abl tyrosine-kinase inhibitor, in K562 cells while treatment with other cytotoxic drugs do not increase ZNF224 levels. By knock-down and over-expression of ZNF224 in K562 cells we also demonstrate that
ZNF224 induction by imatinib is associated with an increase in apoptotic cell death.

Our findings suggest that the increased expression ZNF224 may represent an important event in the drug-induced apoptosis in leukemia cells and may have implications for cancer chemotherapy strategies.

References

SW04.S17–25
Metformin inhibits senescence associated secretory phenotype
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We show that the antidiabetic drug metformin inhibits the expression of genes coding for multiple inflammatory cytokines seen during cellular senescence. Conditioned medium (CM) from senescent cells stimulates the growth of prostate cancer cells but treatment of senescent cells with metformin inhibited this effect. Bioinformatic analysis of genes downregulated by metformin suggests that the drug blocks the activity of the transcription factor NF-kB. In agreement, metformin prevented the translocation of NF-kB to the nucleus and inhibited the phosphorylation of IKB and IKKa/b, events required for activation of the NF-kB pathway. These effects were not dependent on AMPK activation or on the context of cellular senescence, as metformin inhibited the NF-kB pathway stimulated by LPS in ampk null fibroblasts and in macrophages. Taken together, our results provide a novel mechanism for the anti-aging and anti-neoplastic effects of metformin reported in animal models and in diabetic patients taking this drug.

SW04.S17–26
Overexpression of Rukl/CIN85 in breast adenocarcinoma MCF-7 cells results in increased chemoresistance
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Resistance to multiple chemotherapeutic agents is a common clinical problem in the treatment of cancer. Multidrug resistance can result from blockage apoptosis that is activated by most anticancer drugs, activation of coordinately regulated detoxifying systems, and alterations in the cell cycle and checkpoints that renders cells relatively resistant to the cytotoxic effects of drugs on cancer cells. There are data that certain adaptor proteins may be involved in the regulation of these processes. The aim of this study was to investigate the possible role of adaptor protein Rukl/CIN85 that contribute to breast cancer malignancy in the development of breast adenocarcinoma MCF-7 cells chemoresistance.

A measure of metabolic activity of cells treated with cisplatin, doxorubicin, etoposide and tamoxifen revealed that cells with overexpression of Rukl/CIN85 were more resistant to studied drugs than control cells. To investigate possible mechanisms involved in increased chemoresistance of Rukl/CIN85 overexpressing cells the content and activity of ATP-binding cassette membrane transporters were evaluated. Using flow cytometry it was shown that relatively high percentage of MCF-7 cells with the highest expression level of Rukl/CIN85 were positive to ABCG2. The functionality of these transporters was characterized by studying the efflux of such dyes as Rhodamine 123 and Toluidine Blue from treated cells. Obtained results indicated that efficacy of Toluidine Blue and Rhodamine 123 elimination is positively correlated with Rukl/CIN85 expression level. Cells with the highest expression level of Rukl/CIN85 are also characterized by larger number of cells in G0/G1 phases compared to control cells that can correlate with their relatively proliferative quiescence. Overexpression of Rukl/CIN85 also resulted in the constitutive activation of Akt kinase. Interestingly, knocking down of Rukl/CIN85 using specific shRNA lentivirus partially reversed the observed effects of Rukl/CIN85 overexpression on MCF-7 drug resistance. Increasing of MCF-7 cells chemoresistance was shown to be accompanied by enhanced manifestation of such breast cancer stem cells features as the ability to form mammospheres and number of CD44+/CD24− cells.

The data obtained indicate the potential role of adaptor protein Rukl/CIN85 in the development of chemoresistance in breast adenocarcinoma MCF-7 cells.

SW04.S17–27
New insights into the mechanism of action of the glycerophosphoinositols: identification of the tyrosine phosphatase Shp1 as a direct target
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The glycerophosphoinositols (GPIs) are cellular products of phospholipase A2 1Valpha activity on the membrane phosphoinositides. Glycerophosphoinositol (GroPIns) and glycerophosphoinositol 4-phosphate (GroPIns4P) are the most active and well studied of the GPIs. When added exogenously, the GPIs can enter cells and have multiple effects, such as modulation of actin cytoskeleton organisation in fibroblasts, and reduction of invasive potential of metastatic cells [1]. Attempts to identify the molecular targets of the GPIs through a proteomic approach have led to the identification of 70 proteins that are involved in various aspects of cell regulation. Among these, we focussed on Shp1, a well-known regulator of Src activation, as it might be related to the reported signalling pathway leading to GroPIns4P-mediated modulation of the actin cytoskeleton, which involves Src [2]. We studied Shp1 in the context of GroPIns4P-induced membrane ruffle formation in NIH3T3 fibroblasts, where inhibition of enzymatic activity of Shp1 completely abolished GroPIns4P-mediated reorganisation of the actin cytoskeleton. Furthermore, a role for Shp1 has been demonstrated in GroPIns-mediated inhibition of tumour cell invasion. In A375MM melanoma cells, GroPins treatment results in inhibition of extracellular matrix degradation [3], and this activity is suppressed when an inactive mutant of Shp1 is expressed, while it is essentially unaffected by expression of the native enzyme. This approach led to the definition of Shp1 as the first direct GPI target that has been identified to date, thus defining the cascade involved in GPIs-mediated control of the actin cytoskeleton.

References
increasing EGFR molecules was paralleled by decreasing EGFR homoassociation and unexpectedly, increased expression of ErbB1 and integrin molecules remained elevated and defined the extent of radiation resistance. In a flow cytometric study on adhesion molecule expression we have identified that integrin β1 is upregulated upon ectopic expression of excess EGFR in U251 glioblastoma cells. Decreasing radiosensitivity correlated with increasing EGFR and integrin β1 expression levels. While a great excess of ErbB1 provided a colony forming advantage over medium excess, it did not yield better radiation resistance or faster proliferation under regular culture conditions, and clones with a great excess dropped to medium level over time. In the meanwhile, integrin β1 levels remained elevated and defined the extent of radiation resistance. Interestingly, increased expression of ErbB1 and integrin molecules was paralleled by decreasing EGFR homoassociation and increasing EGFR – integrin β1 heteroassociation rates discerned from flow cytometric FRET. A microscopic two-sided FRET approach revealed that in pixels with higher EGFR – integrin β1 heteroassociation, EGFR homoassociation was lower, indicating a competition for association partner between these molecules. Furthermore, the shift towards heteroassociation was accompanied by a boosted Akt phosphorylation response to EGF, a possible explanation for greater radiation resistance in high expressing clones. Inhibition of PI3K reverted the increased radioresistance in high-integrin clones, supporting the idea that the Akt pathway jointly stimulated by EGFR and integrin β1 could be instrumental in mediating radiation resistance.

Treatment of multiform glioblastoma is frequently hampered by decreased radiosensitivity of the tumor. Recent findings provide evidence for functional crosstalk between certain cell adhesion molecules and receptor tyrosine kinases suggesting that they may contribute to therapy resistance. Also, overexpression of EGFR/ErbB1 has been shown to correlate with worse outcome and radiation resistance. In a flow cytometric study on adhesion molecule expression we have identified that integrin β1 is upregulated upon ectopic expression of excess EGFR in U251 glioblastoma cells. Decreasing radiosensitivity correlated with increasing EGFR and integrin β1 expression levels. While a great excess of ErbB1 provided a colony forming advantage over medium excess, it did not yield better radiation resistance or faster proliferation under regular culture conditions, and clones with a great excess dropped to medium level over time. In the meanwhile, integrin β1 levels remained elevated and defined the extent of radiation resistance. Interestingly, increased expression of ErbB1 and integrin molecules was paralleled by decreasing EGFR homoassociation and increasing EGFR – integrin β1 heteroassociation rates discerned from flow cytometric FRET. A microscopic two-sided FRET approach revealed that in pixels with higher EGFR – integrin β1 heteroassociation, EGFR homoassociation was lower, indicating a competition for association partner between these molecules. Furthermore, the shift towards heteroassociation was accompanied by a boosted Akt phosphorylation response to EGF, a possible explanation for greater radiation resistance in high expressing clones. Inhibition of PI3K reverted the increased radioresistance in high-integrin clones, supporting the idea that the Akt pathway jointly stimulated by EGFR and integrin β1 could be instrumental in mediating radiation resistance.

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Abstracts


SW04.S17–28
Molecular interactions of EGFR and integrin beta 1 in glioblastoma cells correlate with Akt mediated radioresistance

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Cancer is one of the most frequent neoplastic diseases in human population and one of the most frequent causes of death. There are a lot of pathological factors, including reactive oxygen species (RVK) involved in the process of cancer initiation and progression. RVK can activate Nuclear transcriptional factor kappa B (NF-kB), which controls genes involved in tumor progression. The matrix metalloproteinase’s generally function to degrade proteoglycans and matrix glycoprotein’s. The aim of the present study was to assess the levels of final lipid peroxidation products TBA-reactive substances (TBARS), quantitative expression of NF-kB and activity of matrix metalloproteinase 9 (MMP-9) in tumor tissue. Investigations were conducted in tissue homogenate of 28 primary colorectal cancers. As a control, the same amount of sample was collected from macroscopically unchanged colon regions of the most distant location to the cancer. Sample was also collected from the closest healthy tissue surrounding cancer. Level of TBARS was determined by method according to Andrewa et al. NF-kB expression was determined by method of indirect immunofluorescence. Activity of MMP-9 was determined by ELISA method using commercial kit manufactured by ‘Anaspec’. Our studies demonstrated a statistically significant increase in the level of lipid peroxidation products (TBARS) compared to control (p < 0.001), and also increase in surrounding tissue level of TBARS compared to control (p < 0.001). Expression of NF-kB was significantly higher compared to control (p < 0.001). Activity of MMP-9 was increased compared to control (p < 0.01), and also the activity in surrounding tissue was increased compared to control (p < 0.05). The highest activity of MMP-9 was in T2 stadium of cancer. These results suggest that colorectal carcinogenesis is associated with oxidative stress, higher NF-kB expression and high MMP-9 activity and that surrounding tissue also has disrupted oxidative status and changes in MMP-9 activity, which may be involved in the process of tumor invasion, considering that T2 stadium had highest activity of this enzyme. This fact may be useful in determining the margins during tumor resection and during postoperative treatment.

SW04.S17–30
Cx43 participates in the pre-selection of metastatic progenitors during prostate cancer metastatic cascade in vitro

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Cx43 participates in the pre-selection of metastatic progenitors during prostate cancer metastatic cascade in vitro

D. Ryszawy1, M. Sarna2, K. Szpak1, M. Rak1, M. Michalik1, M. Siedlar3, E. Zuba-Surma1, W. Korohoda1, Z. Madeja1 and J. Czyz1
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In contrast to ex vivo approaches and the strategies based on genetic engineering, cancer cell lines characterized by phenotypic plasticity give the opportunity to trace the dynamics of clonal evolution of invasive sub-populations. We used such prostate carcinoma cell lines as a tool for scrutinizing channel-dependent and channel-independent involvement of gap junctional protein (GJ) connexin(Cx)43 in the cell recruitment to invasive cancer loci. Invasive AT-2 subsets propagated from the cells predilected to transmigrate narrow obstacles in chemokinetic conditions, displayed elevated Snail-1 and connexin(Cx)43 expression levels. Expression profiles of EMT markers in established epitheloid and fibroblastoid AT-2 sub-clones indicated the involvement of Snail-1 in incomplete EMT-related shifts, which determine cell invasive-ness and phenotypic AT-2 heterogeneity. Cx43 and Snail-1 levels were increased in fibroblastoid sub-clones, whereas Snail-1 silencing in fibroblastoid AT-2 cells evoked Cx43 down-regulation paralleled by their phenotypic transition toward epitheloid phenotype. Corresponding phenotypic shifts occurred upon Cx43 silencing and coincided with Snail-1 down-regulation. These correlations, not observed in epitheloid AT-2 and DU-145 cells, implicate reciprocal links between Snail-1- and Cx43-dependent mechanisms in functional speciation of prostate cancer cells. Cx43 may directly predispose post-EMT cells for local invasion in a GJ channel-independent manner, whereas their competence for GJ coupling with stromal tissue may further facilitate metastasis. When extrapolated to in vivo situation, these data situate Cx43 function in the context of clonal evolution model of prostate can-
Regucalcin (RGN) is a calcium (Ca²⁺)-binding protein playing an essential role in the maintenance of intracellular Ca²⁺ and oxidative stress levels, and also in the control of cell proliferation and apoptosis. Cancer arises on the deregulation of the aforementioned processes, and curiously our previous work demonstrated that RGN expression is diminished in human breast cancer and correlated with tumour grade. This work aims to investigate whether RGN underexpression may be associated with malignant transformation of breast. Virgin female transgenic rats overexpressing RGN (Tg-RGN, n = 31) and wild type (Wt, n = 8) controls received a single intragastric administration of the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA, 20 mg/kg in 0.2 ml sesame oil). Two weeks after, animals were weekly inspected and euthanized when palpable tumor(s) were ≥2 cm or 44 weeks after DMBA administration. Mammary gland tumors were processed for histological analysis and classified as precancerous lesions, and non-invasive or invasive tumours. Although the latency period (14 weeks) was identical in Tg-RGN and Wt, 44 weeks after experiment began tumor incidence was significantly different between groups (p < 0.0001); 100% of Wt versus only 25.8% of Tg-RGN rats developed a tumor. Moreover, histological analysis (p < 0.0043) showed that the majority of tumors in both Tg-RGN (76.92%) and Wt (54.54%) groups is non-invasive forms. However, the percentage of invasive tumors in Wt (45.5%) animals is significantly higher than that observed in Tg-RGN (3.84%). Noteworthy precancerous lesions (19.23%) in Wt (45.5%) animals is significantly higher than that observed in Tg-RGN (3.84%). The obtained results reveal that Tg-RGN animals are less susceptible to develop mammary gland tumors, and resistant to the acquisition of more aggressive phenotypes. These findings indicate a protective effect of RGN in breast carcinogenesis. Expression analysis of cell cycle and apoptosis regulators is underway to determine the mechanisms behind RGN control of breast cells growth.

**SW04.S17–31**

Transgenic rats overexpressing regucalcin display lower-susceptibility to develop DMBA-induced mammary gland tumors

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Increased rate of aerobic glycolysis compared to normal tissues. He identified large amounts of glucose and lactate even in the presence of oxygen in tumors (the Warburg phenotype of cancer cells). Glycolysis and oxidative phosphorylation are the metabolic pathways associated with energy provision during cell cycle. Glycolysis, one of the most ancient metabolic processes (approximately 3.5 billion years), is a relatively low-energy providing pathway compared to oxidative phosphorylation. But increase in glycolysis is one of the main features of energy metabolism in most proliferating cells including cancer cells. At the same time, several glycolytic genes are highly expressed in all tissues and thus considered as housekeeping genes (such as GAPDH). We have studied the transcriptomic data of human renal, lung and breast cancers and estimated the tissue and cancer specific glycolytic expression patterns. We have shown that in all studied neo-plasms even the ‘core’ glycolytic genes were differentially expressed in comparison to normal tissues. Among these genes we describe the overexpression of PKFP, ALDOA and GAPDH in renal cancer, significant upregulation of HK2 in breast cancer and GPI in squamous cell lung cancer. Several genes, such as ALDOA, ENO1, GAPDH, PGK1, were characterized by an unstable expression level with high variation in both normal and tumor samples. We have selected the subgroups of the probable housekeeping (reference) glycolytic genes for these cancers: HK1, ADPGK, GPI, PKM2 for renal cancer; ADPGK, ALDOA, GAPDH, PKG1, BPGM, ENO1, PKM2 for squamous cell lung cancer and ADPGK, BPGM, ENO2, GI, PFKM for breast cancer. The cases of decrease of glycolytic enzyme’s mRNA level were rare. These data were validated using real-time PCR. Therefore, we have detected dysregulation of glycolysis in all studied cancers.

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**SW04.S17–33**

Genetic variations associated with coronary restenosis in Kazakh population

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Restenosis is a main adverse event of percutaneous coronary intervention (PCI) widely used to treat coronary heart disease (CHD). Inflammation, smooth muscle proliferation and local thrombosis are considered as the main mechanisms for its development. In this sense, inflammation-related genes are potential genetic factors influencing the risk of restenosis. The purpose of the present case-control study was to evaluate whether different genetic determinants are involved in the formation of restenosis in the Kazakh population. The study included 75 patients diagnosed with restenosis after PCI, 34–79 years old (mean age ± 55 years, male/female distribution − 70:5). Control group included 150 cases with CHD who have not developed restenosis within 6 months after intervention. Individuals from both the case and control groups were ethnic Kazakhs. Genotypes of polymorphic loci of IL-10, TNF-α and VDR genes were determined by real-time PCR with Taqman probes using CFX96 thermal cycler (Bio-Rad, USA).

Odds ratios and p-values have been calculated for all studied SNPs; interleukin 10 −1082G/A (rs1800896; OR = 0.823, CI = 0.238–2.395, p = 0.39); interleukin 10 −819T/C (rs1800871; OR = 0.91, CI = 0.572–2.247, p = 0.67); interleukin 10 −592A/C
and receptor D with human stomach tumours sections in IHC. Those new expressing cell lines (HeLa, HEK293T). The antibodies also react with synthetic peptide mimotope (conjugated with ovalbumin) was used in immunofluorescence (IHC) and flow cytometry (FACS). A computational method was used to predict antigenicity and define few mimotopes which is based on silico group of patients with the stomach tumors. We had performed genetic analysis to understand the importance of nitric oxide in the progression of human colon carcinoma T. A. Rinaldi¹, F. T. Ogata¹, T. Salo² and H. P. Monteiro¹

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Nitric oxide (NO) is a signaling free radical that is produced both by tumor cells and host cells. Two constitutive – endothelial (eNOS) and neuronal (nNOS) – and one inducible (iNOS) isoform of the NO synthase produces NO upon physiological and pathophysiological stimuli. We used as experimental model human colon carcinoma cell lines derived from the same patient, SW 480 was obtained from the primary tumor, and SW620 from the lymph node metastatic site. To assess the role of nitric oxide in colon cancer progression, iNOS was permanently silenced using shRNA in the metastatic human cell line SW620. The clone obtained (SW620-I12) when compared to its parental cell line displays lower growth rates, diminished expression of genes associated with angiogenic, proliferating, hypoxia and multidrug resistance, and also overexpression of p21Waf, a tumor suppressor gene, and is positive for e-cadherin expression, on contrary to its parent cell line. Taken together, the data suggests that SW620-I12 resembles the primary tumor SW480 in many ways and corroborates with the importance of iNOS and nitric oxide generation for the development of colon carcinoma cells.

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SW04.S17–36
Dioxin receptor modulates fibroblast adhesion and migration through Cbp-Csk-Src and fibronectin combined pathways to control beta1 integrin activation
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The Aryl hydrocarbon Receptor (AhR) was firstly described due to its critical role in xenobiotic-induced toxicity and carcinogenesis. However, recent studies have suggested a physiological regulatory role for the dioxin receptor (AhR) in several aspects of cell adhesion and migration. Following our previous work, we report here that these cell functions are, in part, driven by the C-terminal Src kinase-binding protein (Cbp) signaling pathway, which controls Betal integrin activation, and that this mechanism is AhR dependent.

T-FGM AhR−/− fibroblasts displayed higher integrin Betal activation, revealed by increased binding of the activation reporter 9EG7 anti-Beta1 mAb and of a soluble fibronectin fragment, as well as by enhanced Talin- betal association. AhR−/− fibroblasts also showed increased Fibronectin secretion and impaired directional migration and wound healing response.

Notably, interfering Cbp expression in AhR−/− fibroblasts reduced Betal integrin activation, improved cell migration and rescued wild-type cell morphology. Cbp over-expression in T-FGM AhR−/− cells enhanced the formation of inhibitory Csk-Cbp complexes which in turn reduced c-Src p-Tyr416 activation and focal adhesion kinase (FAK) phosphorylation at the c-Src-responsive residues p-Tyr576 and p-Tyr577. The c-Src target and migration-related protein Cav1 was also hypophospho-
orylated at p-Tyr14 in AhR−/− cells, and such effect was rescued by down-modulating Cbp levels.

Thus, AhR regulates fibroblast migration by modulating Beta1 integrin activation via Cbp-dependent, Src-mediated signalling.

**SW04.S17–37**
The biochemistry and signaling of bioactive lipids of N-acyl dopamines group

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N-Acyl dopamines (NADA) are biogenic conjugates of dopamine with long chain fatty acids. For the first time, the ability of animal tissue homogenates to acylate biogenic amines with fatty acids was discovered in mid-XX century in rat brain, kidneys and liver. This research was considered secondary to the studies of protein biosynthesis and thus didn’t draw much attention. However, after the discovery of arachidonic acid ethanamide as an endogenous ligand of cannabinoid receptor in 1992, the interest towards reappeared, and the dopamine amides of polyunsaturated fatty acids were chemically synthesized in Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry as possible ligands or blockers of this receptor. The intense research of NADA led in 2002 to the discovery of their being endogenous ligands of vanilloid receptor TRPV1. Today, NADA were found ligands or blockers of this receptor. The intense research of NADA led in 2002 to the discovery of their being endogenous ligands of vanilloid receptor TRPV1. Today, NADA were found ligands or blockers of this receptor.

The targets of NADA are vaniloid receptor TRPV1. The present study might provide a basis for creating a general pharmacophore model to design and develop new ATP-noncompetitive agents with chemopreventive or chemotherapeutic potency.

**SW04.S17–38**
A chrysin derivative suppresses EGF-induced anchorage-independent growth of mouse epidermal JB6 P+ cells by inhibiting Cdks


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Chrysin (5,7-dihydroxyflavone), a natural flavonoid widely distributed in many plant extracts, honey and propolis, has chemopreventive properties against various cancers. However, the anticancer activity of chrysin observed in in vivo studies has been disappointing. Here, we report that a modified chrysin, compound mc-1, more strongly prevented EGF-induced neoplastic transformation of JB6 P+ cells compared to chrysin. It significantly blocked cell cycle progression of EGF-stimulated cells at the G1 phase and inhibited the G1/S transition. It also caused the loss of Rb phosphorylation at both Ser795 and Ser607/811, the preferred sites phosphorylated by CDK4/6 and CDK2, respectively. IP-kinase assay results showed that mc-1 attenuated endogenous CDK4 and CDK2 kinase activities in EGF-stimulated JB6 P+ cells. Pulldown and in vitro kinase assay results indicated that mc-1 directly binds with CDK2 and CDK4 in an ATP-independent manner and attenuated their kinase activities. A crystal structure of CDK2 was used to build a binding model between mc-1 and CDK2 and it predicted that mc-1 was located inside the CDK2 allosteric binding site. The binding was further verified by a protein point mutation binding assay. Our results indicated that mc-1 is an ATP-noncompetitive CDK inhibitor with antitumor promoting effects, which acts by binding inside the CDK2 allosteric pocket. The present study might provide a basis for creating a general pharmacophore model to design and develop new ATP-noncompetitive agents with chemopreventive or chemotherapeutic potency.

**SW04.S17–39**
Acceleration of tumor growth by nitric oxide production in macrophages after radiotherapy

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Radiotherapy is a widely used local treatment for malignant tumors, characterized by uncontrolled growth, adjacent tissue-invasion and metastasis. However, recurrence is a challenging limit to the application of radiotherapy to tumor treatment. Our experimental observations indicate that the irradiated tumor-bearing host exerts tumor-promoting effects. Here, we focused on direct communication between macrophages and tumor cells in tumor micronenvironment (TME) that lead to invasion and egress of tumor cells into the blood vessels in response to g-irradiated cancer cells. Our data showed that in vivo growth of CT-26 (H-2b) mouse colon carcinoma and in vitro production of nitric oxide from macrophages were increased by g-irradiated CT-26 cells in comparison with those by non-irradiated control cells.

Stimulation of macrophages (H-2b) with g-irradiated CT-26 cells increased NO production and gene expression of nitric oxide (NO), inducible nitric oxide synthase (iNOS) and toll-like recep-

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Abstracts

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tor 1 (TLR1). While TLR1 overexpression increased NO production, it was reduced by the inhibition of TLR1 expression with TLR1-siRNA. In vivo tumor growth was reduced by the administration of N\(^\cdot\)\(^\cdot\)L-monomethyl-L-arginine (L-NMMA) and it was also decreased by the transplantation of peritoneal macrophages exuded from tumor-bearing L-NMMA-treated mice. Taken together, data demonstrate that tumor growth might be relapsed by the production of NO through TLR1-mediated responses of macrophages to g-irradiated cancer cells. It suggests for the first time that NO from macrophages could be a useful target for the regulation of radiotherapeutic responses by g-irradiated tumor cells.

**SW04.S17–40**

**Subchronic treatment with ferric nitrilotriacetate (FeNTA) induces AP-1 activation and cyclin D1 overexpression: possible mechanisms of renal carcinogenicity**

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Renal cell carcinoma (RCC) represents almost 90% of cancer arising from adult kidney. This cancer is asymptomatic, and initial diagnosis generally occurs in advanced or even in metastatic stages. Early stages detection or studies are almost impossible in patients. Ferric nitrilotriacetate (FeNTA) induced RCC is a useful model to analyze early events on RCC development for this goal. Chronic intraperitoneal FeNTA injections give rise to RCC in rats, and its administration along 1 or 2 months leads to renal dysplasia. Oxidative stress is involved in FeNTA carcinogenicity, but the tie molecular mechanisms remain unclear. Cyclin D1 and c-Jun has been proposed to participate in RCC development, and both molecules respond to oxidative stress. In the present work we found that less advanced FeNTA induced tumours show high nuclear p-c-Jun levels, compared to normal tissues, and this alteration diminishes or disappear in more advanced tumours, suggesting that c-Jun may participate in early developmental stages of FeNTA induced RCC. To test this hypothesis we designed sub-chronic FeNTA schemes during 1 and 2 month of FeNTA treatment. Subchronic FeNTA administration induced renal overexpression and phosphorylation of c-Jun, an increase of AP-1 activity and high levels of cyclin D1 protein. These changes were more severe at 2 months compared to 1 month protocol. These alterations were not induced in liver or lungs. We concluded that AP-1 hyperactivation and cyclin D1 overexpression may be related to FeNTA carcinogenicity.

**SW04.S17–41**

**Effects of nuclear receptor HNF4\(\alpha\) repression in human pancreatic ductal adenocarcinoma cells**

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HNF4\(\alpha\) transcription factor belongs to the family of hepatocyte nuclear factors (HNFs) that are essential for differentiation and functionality of liver, pancreas, intestine and kidney. HNF4\(\alpha\) is a key regulator of HNF transcription control network in hepatocytes. Derangement of HNF4\(\alpha\) expression is crucial for the development and progression of hepatocellular carcinoma (HCC); restoration of HNF4\(\alpha\) expression in HCC cells results in partial reversion of their malignant properties. HNF4\(\alpha\) acts as a tumor suppressor in kidney and intestinal epithelium cells, but its function in pancreatic cells is poorly investigated.

HNF4\(\alpha\) is expressed as two groups of isoforms driven by alternative promoters, P1 and P2, and differed by distinct expression pattern in various tissues and trans-activation ability. In normal pancreatic tissue HNF4\(\alpha\)P2 isoforms are predominant. We have shown that HNF4\(\alpha\) expression is often deregulated in pancreatic ductal adenocarcinoma (PDAC) tissue samples and cell lines. Extrinsic HNF4\(\alpha\)P1 isoforms are activated in moderately differentiated cells while poorly differentiated cells lack the expression of all HNF4\(\alpha\) isoforms. To find out the role of HNF4\(\alpha\) deregulation in PDAC progression we have studied the biological impact of shRNA-mediated HNF4\(\alpha\) knock-down in moderately differentiated PDAC cell line AsPC1.

HNF4\(\alpha\) knock-down in AsPC1 cells resulted in diminished expression of HNF1\(\alpha\), direct target gene of HNF4\(\alpha\) and important regulator of pancreas differentiation. Cells with repressed HNF4\(\alpha\) expression demonstrated increase in their ability for directional migration and induction of N-cadherin expression. On the other hand, AsPC1-shHNF4\(\alpha\) cells exhibit lower colony formation ability and significant decrease in proliferation and DNA synthesis.

These results indicate that pancreatic carcinogenesis is often accompanied by deregulation of HNF4\(\alpha\) expression. HNF4\(\alpha\) repression in PDAC cells exerts complex alterations of biological properties of PDAC cells, which include both promotion and attenuation of certain cell characteristics that determine the degree of malignant phenotype manifestation.

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and over-expression of this phosphatase promotes apoptosis. Our data also show that the mechanism of action of DUSP1 is based on the inhibition of both the NF-kB and p38 MAPK signalling pathways. First, we show that DUSP1 decreases NF-kB dependent transcription by impairing the translocation of p65/NF-kB to the nucleus. Moreover, DUSP1 impairs TNF-alpha-induced p38 MAPK activity, and experiments using a specific inhibitor show that p38 MAPK blockade exerts the same effects than DUSP1 over-expression on both apoptosis and NF-kB activity. Consistently, DUSP1 promotes apoptosis and decreases NF-kB activity in cells in which p38 MAPK is induced by TNF-alpha treatment. Overall, our results provide evidence for a role of DUSP1 in the apoptosis of prostate cancer cells, through a mechanism involving the inhibition of both p38 MAPK and NF-kB.

SW04.S17–43

What myosin VI does in the neuromuscular junction, sarcoplasmic reticulum and muscle nuclei?

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Myosin VI (MVI) is a unique motor protein that moves on actin filaments in the opposite direction to all other myosins. As a cargo transporter and/or anchor MVI is engaged in numerous processes associated with actin cytoskeleton such as cell migration, endocytosis and intracellular trafficking as well as gene transcription. The function of MVI in muscle tissue is not unknown. Therefore, we addressed for the first time MVI function in striated muscle by examining its expression and distribution in rat hindlimb skeletal muscle. Our studies showed that MVI was associated with membranous structures such as sarcoplasmic reticulum and the neuromuscular junction, it was also present within the muscle nuclei. The presence of MVI in sarcomplasmic reticulum was also observed in mice cardiac muscle. Interestingly, the defined distribution pattern of MVI in skeletal muscle was abolished in denervated muscle and was accompanied by significant increase in its amount in the muscle fiber.

In addition, we have identified several novel potential MVI-binding partners, which seem to aid our observations that in striated muscle MVI could be involved in postsynaptic trafficking as well as in maintenance of and/or transport within the sarcoplasmic reticulum and non-sarcomereic cytoskeleton.

SW04.S17–44

Targeting ovarian cancer at the molecular mechanisms level of the treatment efficiency increase

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Patients are often prescribed neoadjuvant polychemotherapy (NPChT) to treat ovarian cancer (OC) at the late stages (III-IV) development process. After the NPChT it becomes possible to make an operation to delete the original tumor in (OC) patients. We made diagnostic complex of biochemical, clinical and ultrasonic criteria for NPChT efficiency evaluation. It helped to find optimal number of NPChT courses for every patient and improve the efficiency of treatment. It was found that the changes in level of total glycosaminoglycans (GAG) in blood serum up to 75–70%, chondroitinsulphate up to 80–65%, II GAG- fraction up to 75–65% of the original, I and III fractions, ratios of total GAG and their fractions, II-1β, II-4, II-6, TNFα, INFγ to normal data, as well as the content of CA 125 to 9.6–3.6% of its initial value are markers of making operation possibility in patient having OC stages III-IV. We suppose that structural degradation of ECM appears in the epithelial tissues of patients during the OC- development. It effects the GAG spectrum in blood serum and evidently precedes invasion and tumor prevents invasion and metastasis that effects the GAG spectrum in the blood metastasis. Great number of NChT courses modules neoplastic activity best of all and serum of patients. Molecular mechanisms of increase for OC treatment efficiency are discussing.

SW04.S17–45

Functional analysis of FOXO3a-p53 interaction in Mantle Cell Lymphoma cells

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Mantle cell lymphoma (MCL) is a subtype of B-cell non-Hodgkin lymphoma characterized by the (t11:14) chromosomal translocation, which results in aberrant expression of cyclin D1. This genetic event is present in virtually all cases of MCL, whereas additional genetic alterations that occur in subsets of MCL have been described. Most of these alterations appear to disturb the cell cycle machinery or interfere with the cellular response to DNA damage.

FOXO3a is a potent transcriptional activator that triggers the expression of a program of genes involved in cell cycle arrest, DNA repair, hypoxia response and apoptosis. FOXO3a is the predominant member of the FOXO subfamily in lymphoid tissues, and is essential for proliferation of cells of the immune system. Interestingly, it plays an essential role in lymphomagenesis, particularly in the maintenance of the haematopoietic stem cell pool. FOXO transcription factors are negatively regulated by the PI3K-Akt pathway. Genetic studies have revealed that members of the PI3K pathway such as PI3KCA or Akt1 are over-expressed in MCL.

Several nodes of interaction have been identified between FOXO transcription factors and p53, a major tumor suppressor in humans and mice. We have evaluated the relative role of the FOXO3a-p53 axis in a panel of MCL cell lines with different p53 status. We have analysed the response of MCL cells to chemical modulators of this pathway such as Nutlin-3a, Psammaplysene A and DNA damaging agents. Cell viability assays have been conducted in the panel of cell lines, and the expression levels of FOXO3a, p53 and its targets have been analysed in response to such agents. FOXO3a is activated in response to Nutlin-3a, and expression of the FOXO target FOXM1 is increased. Also, colocalization and coimmunoprecipitation studies show evidence of FOXO3a-p53 binding in MCL cells. Interestingly, this binding is modulated by DNA damage. Taken together, our results suggest the importance of the interaction between FOXO3a and p53 in MCL biology.
SW04.S17–46
Targeting MyosinVa as a strategy to prevent cellular export of methotrexate in melanoma
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Human melanoma is a significant clinical problem because it is resistant to treatment by most chemotherapeutic agents, including antifolates. Melanosomal sequestration and cellular exportation of cytotoxic drugs have been proposed to be important melanoma-specific mechanisms that contribute to the resistance of melanoma to these agents. In particular, the treatment of melanoma with methotrexate (MTX) alters melanosogenesis and accelerates the exportation of melanosomes. However, the processes by which MTX is trapped into melanosomes and exported out of cells have not been elucidated. In this study, we identified MyosinVa (MyoVa) as a possible mediator of these cellular processes. The results demonstrated that melanoma treatment with MTX leads to Akt2-dependent MyoVa phosphorylation, which enhances its ability to interact with melanosomes and accelerates their exportation. Due to these findings, we designed a MTX combination therapy to increase the susceptibility of melanoma to this drug by blocking the MyoVa/Akt2 pathway. Because 7-hydroxystaurosporine (UCN-0) has been shown to potently inhibit PDK1, which activates Akt by phosphorylation, we hypothesized that the inhibition of Akt2 phosphorylation by UCN-01 may result in the disruption of MTX-stimulated melanosome transport. By avoiding MTX export, we observed that the E2F1 apoptotic pathway is functional in melanoma, and its induction activates p73 and Apaf1 following a p53-autonomous pro-apoptotic signalling event. In vivo studies in mice also indicated that low doses of UCN-01, when combined with MTX, produced a marked reduction for not only tumour growth but also melanoma metastasis. In summary, we observed that the combination of MTX and UCN-01 may represent a therapeutic option for the treatment of this invasive disease.

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SW04.S17–47
Prognostic significance of circulating tumor cells in castration resistant prostate cancer
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Background: The advanced stage of prostate cancer called castration resistant prostate cancer (CRPC) is an incurable disease with median survival around 15 months. More than 85% of patients suffering from CRPC exhibit metastases, mostly in bone. Due to the ongoing metastatic process we are able to detect circulating tumor cells (CTCs) in the blood of the majority of the patients with CRPC. We aimed at the detection of CTCs in the blood of patients with CRPC together with their characterization a study of their behavior during in vitro cultivation.

Methods: Five millilitres of peripheral blood of patients with CRPC was used for immunomagnetic separation of CTCs using AdnaTest ProstateCancerSelect kit (Adnagen, Langenangen, Germany). mRNA was isolated from enriched fraction of CTC using AdnaTest ProstateCancerDetect kit (Adnagen, Langenangen, Germany). Reverse transcription was performed for generating cDNA form mRNA, which was subsequently used as a template for multiplex PCR. Amplification of at least one of three tumor-associated antigens (PSA, PSMA, EGFR and control gene β-actin) proved presence of CTC in samples. The evaluation of the results was performed with Bioanalyzer 2100 (Agilent). Peripheral blood was drawn twice: at the time of CRPC diagnosis and after the first four cycles of docetaxel chemotherapy. Another five millilitres of the peripheral blood of the patients was used for isolation of layer of the mononuclear cells by gradient centrifugation. CTCs should be present in this fraction of blood. By cultivating these collected cells we tried to reach growing population of CTCs. We tested this method by using cells of LNCap cell line in the blood of healthy donor instead of CTCs.

Results: Currently, we tested 21 patients with CRPC at the time of diagnosis and 16 of them also after first four cycles of docetaxel therapy. At the time of CRPC diagnosis 85% of the patients were CTCs positive. After the docetaxel therapy only 44% of the patients remained CTCs positive and 95% showed decrease of the monitored markers. Although we succeeded in the cultivation of LNCap cells isolated from blood, with the patient's samples we did not achieve the same results yet.

Conclusions: Our results indicate that most of the patients with CRPC have CTCs present in their blood at the time of diagnosis. In our opinion the level of CTCs and it’s changes during the therapy could serve as a prognostic and therapy efficiency marker for the clinicians. Moreover, the expression of the tumor-associated genes in CTCs differs between the patients. For this reason, we are estimating that CTCs could give useful information about differences between the patients with CRPC regarding to specific antigens expression for targeted therapies or chemotherapy sensitivity for personalized medicine. Successful cultivation of the CTCs should serve as a source of information about biological behaviour of tumor cells.

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SW04.S17–48
V600E BRAF decreases E-cadherin expression through a snail-dependent mechanism in thyroid cancer cells
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BRAF is a main oncogene in human thyroid cancer. Here, we show that BRAF depletion by siRNA or inhibition of its activity by treatment with BRAF inhibitor PLX4720 decreases migration and invasion in thyroid cancer cells expressing oncogenic v600E BRAF through a MEK/ERK-dependent mechanism, since
treatment with the MEK inhibitor U0126 exerts the same effect. Moreover, over-expression of V600E-BRAF increases migration and invasion of wild-type BRAF thyroid cells. Using the same strategies, we demonstrate that these effects are mediated by upregulation of the transcriptional repressor Snail with a concomitant decrease of its target E-cadherin, both hallmarks of EMT. These results reveal a novel V600E-BRAF-induced mechanism in thyroid tumours progression and provide a rationale for using the PLX4720 inhibitor to target V600E-BRAF signalling to effectively control progression of thyroid cancer.

SW04.S17–49
The tumorigenic role of Low Molecular Weight Phosphotyrosine-Phosphatase (LMW-PTP)
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Previous results obtained with a wide array of human carcinomas indicate a significant increase in the expression of LMW-PTP in tumor tissue. A higher expression of LMW-PTP is significantly correlated with worst prognosis and reduced survival (especially for colon cancer). Experiments in animal models for colorectal tumors, confirmed these results. In order to further investigate this topic, we utilized microarray analysis of transcriptome on human MCF10A (mammary epithelial cells), in which expression of LMW-PTP was modulated with transfection, leading to enhanced ectopic expression of the protein. The vast majority of phenotypic changes are associated to the coordinate activation of several genes in a pathway. We then performed pathway analysis scoring for significant mRNA enrichment of genes present in a given pathway (DEGs, differential expressed genes). The combination of the results of statistically significant pathways was used as a descriptor of every single experiment. In this way we identified several transcripts that present marked differences in the level of expression due to LMW-PTP overexpression. Expression pathway analysis strongly suggests an involvement of LMW-PTP in the negative modulation of apoptosis, giving a possible explanation of its tumorigenic effect. LMW-PTP overexpression, in fact, modulate the expression of many different genes involved in the regulation of apoptosis but also in proliferation, migration and angiogenesis. These findings were also corroborated by in vitro experiments on MCF10A cells transfected with a LMW-PTP expressing vector. The escape from anoikis is a crucial step in cell malignant transformation and a key hallmark of metastatic cancer cells: detachment-induced anoikis assay confirmed that ectopic LMW-PTP overexpression causes resistance to apoptosis. Similar results were obtained using Intratoced Hydrochloride or Fluorouracil for induction of apoptosis. Moreover, we performed a test of growth in soft agar: ectopic overexpression of LMW-PTP increases MCF10A colony formation in soft agar. All these results strongly suggests that LMW-PTP overexpression, a phenomenon frequently associated with tumorigenesis, may acts as an inhibitor of apoptosis.

SW04.S17–50
GRP78 a mediator of intrinsic and extrinsic tumor resistance
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Cancer cells and their microenvironment adapt to genotoxic stress by induction of the GRP-78/BiP (glucose-regulated protein 78 immunoglobulin heavy chain binding protein) a chaperone protein of the endoplasmatic reticulum with antiapoptotic properties. In particular GRP78 expression is elevated in tumor cells that grow in a microenvironment characterized by glucose deprivation, acidosis and hypoxia, i.e. in viable tumor cells bordering necrotic regions created by therapy. These cells generally are highly chemo-resistant and protected against lysis by cytotoxic T-cells. Moreover, highly metastatic cells express GRP78 ectopically on the cell surface making cells insensitive to TGF beta signaling. In human cancers elevated GRP78 expression generally correlates with higher pathological grade, recurrence and poor patient survival in breast, prostate and colon cancer. Hitherto, effects of GRP78 exposed on the cell surface or released by tumor cells are poorly understood in context to the respective microenvironment, i.e. immune resistance and resistance to antiangiogenic drugs. Based on the previous work on the role of tumor-derived GRP78 on anti-angiogenic therapies we plan to perform detailed and novel investigations within this research consortium, to gain a profound knowledge on the role of GRP78 in intrinsic and extrinsic tumor resistance of solid and hematological tumors. Thus, we want to establish an inducible knock-down system of GRP78 gene expression in therapy-resistant solid tumor cells to restore sensitivity to anticancer drugs. Different siRNA sequences were tested on MDA-231-MB, HRT-18 and PC-3 tumor cells and GRP78 expression analyzed on mRNA and protein level. We were able to show that GRP78 knock-down was inducing apoptosis in all tumor cell lines tested. Future we established an inducible lentiviral system for overexpression of soluble GRP78 in myeloma cell lines to study mechanisms of intrinsic and extrinsic cancer resistance.

SW04.S17–51
Enhanced levels of asymmetric dimethylarginine in serum of myelodysplastic patients overloaded with iron
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Nitric oxide (NO) was identified as an endogenously produced free radical mediator of endothelial-dependent relaxation. The major sources of NO in vivo are the NO synthase (NOS) isoforms. iNOS expression is enhanced in myelodysplastic syndrome (MDS). Major causes of impairment of the NOS pathway are endogenous inhibitors of NOS: asymmetric dimethylarginine (ADMA) and N⁵-monomethyl-L-arginine (MMA). Since reactive oxygen and nitrogen species (RONS) may increase intracellular ADMA levels, this is a potential positive feedback mechanism to perpetuate increased oxidative stress. The aim of this work was to evaluate the levels of ADMA on the background of oxidative stress and iron overload. The serum samples were obtained from MDS patients (n = 20, age median 43.7) overloaded with iron and from healthy donors (n = 16, age median 44.3). The concentration of ADMA, MMA, SDMA, and homoarginine were obtained using LC-MS/MS method on HILIC column Luna Silica. Malondialdehyde concentrations were evaluated by sample derivatization with thiobarbituric acid with subsequent HPLC analysis with UV detection at 532 nm.

We found significant differences of ADMA (0.41 M, p = 0.0022), MMA (0.067 M, p = 0.033), and SDMA (0.265 M, p = 0.036) as compared with healthy controls (ADMA 0.28, MMA 0.052 M, and SDMA 0.208). MDA concentrations in group of MDS patients (0.77 M) were significantly different from MDA concentrations in healthy control (0.52, p = 0.001).

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The concentrations of NOS inhibitors and markers of oxidative stress (MDA) were significantly elevated in patients with MDS as compared with controls. Despite known enhanced expression of iNOS in MDS patients, we previously observed decreased NO concentrations explainable by impairment of NOS function by its endogenous inhibitors – methylated arginines, that further enhance oxidative stress. Thus, estimation of endogenous NOS inhibitors could be an promising diagnostic mean to follow the MDS progression.

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SW04.S17–52
Caffeine enhances cisplatin efficacy by cell cycle modulation
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Caffeine has been shown to enhance the efficacy of chemother-apy, but the mechanism is not well understood. In this study, we investigated cell-cycle modulation induced by caffeine in combination with cisplatinum treatment of human cancer cells. Proliferation inhibition was determined with a clonogenic assay. Mitotic and apoptotic changes were observed by imaging of 143B dual-color cells, in which GFP is expressed in the nucleus and RFP in the cytoplasm. Modulation of the cell-cycle in combination with cisplatinum was observed using time-lapse imaging analyses of HeLa cells expressing a fluorescent cell-cycle indicator, FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator). The clonogenic assay showed that caffeine increased the efficacy of cisplatin on inhibition of cell proliferation. Dual-color imaging demonstrated that cisplatin decreased mitosis and induced apoptosis in 143B cells. The combination of cisplatin and caffeine reduced the decrease of mitosis and increased apoptosis. Time-lapse FUCCI imaging showed that cisplatin strongly induced cell-cycle arrest at the S/G2/M phases in HeLa cells. Caffeine released the cell-cycle arrest induced by cisplatin, resulting in increased cell-killing efficacy. These data indicates that caffeine can effectively modulate the cell-cycle in cancer cells treated with cisplatin and possibly other drugs.

SW04.S17–53
Intracellular prostaglandin E2 behaves as a pro-metastatic factor in human prostate cancer
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Prostaglandin E2 (PGE2) is frequently increased in prostate cancer and other cancers by increased cell proliferation and migration. Non-steroidal anti-inflammatory drugs are considered to have chem-preventive effects on this disease by reducing the biosynthesis of PGE2 via their inhibition of cyclooxygenase-2, but side effects limited their application, so new approaches are needed to the therapy of this disease.

Here we first investigated whether PGE2 plays an important role in the carcinogenesis of human prostate cancer (androgen-independent) PC3 cell line and found that: i) PGE2 increases cell proliferation ii) decreases cell adhesion iii) increases cell migration iv) promotes angiogenesis and invasion.

SW04.S17–54
Methioninase-induced S/G2-phase-trapping indicated by color-coded imaging for subsequent effective chemotherapy
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Methionine-dependence of cancer cells may be due to excessive methylation reactions in cancer cells. Deprivation of methionine α, γ lyase (methioninase or METase) selectively arrests cancer cells during late S-phase, where they are highly sensitive to DNA-damaging chemotherapy. Fluorescent ubiquitination-based cell cycle indicator (FUCCI), was used to monitor the onset of the S/G2-phase block due to methionine deprivation effected by METase. The S-phase-blocked cancer cells fluoresced yellow or green, in contrast to cancer cells in G1 which fluoresced red. Cancer cells, synchronously blocked in S-phase by METase and identified by their yellow-green fluorescence, were administered DNA-damaging chemotherapy drugs such as doxorubicin, cisplatin, or 5-fluorouracil. Treatment of cancer cells with drugs only without methioninase-effected S-phase synchrony, led to the majority of the cancer cell population being blocked in G0/G1 phase (red fluorescent) where they were resistant to the drugs. In contrast, METase treatment, followed by chemotherapy when FUCCI indicated the S/G2 block was highly effective for killing cancer cells. Color-coded chemotherapy, whereby the cell cycle of cancer cells is selectively and synchronously blocked in S-phase as identified by fluorescent reporters, may be a general approach to effective cancer treatment.

SW04.S17–55
Poly (adenosine diphosphate-ribose) polymerase-1 Val762Ala polymorphism in Turkish gastrointestinal cancer patients
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PARP-1 plays important role in the BER (base excision repair) and maintenance of genomic integrity. Val762Ala genetic variant in the PARP-1 gene contributed to susceptibility of some cancers and decreased PARP-1 enzyme activity in response to oxidative damage. Colorectal cancer is the second most frequent cause of cancer-related death.

A total of 80 paraffin-embedded colorectal cancer specimens were obtained from department of pathology in Cerrahpasa anti-
SW04.S17–56
Expression of GRHL genes in human non-melanoma skin cancers

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Grainyhead-like proteins (GRHL) constitute a highly conserved family of transcription factors whose structures have been conserved in the course of evolution of multicellular organisms. In mammals there are three GRHL genes, present on different chromosomes, which are expressed in a tissue- and spatio-temporally-specific fashion. The GRHL factors are critical for development and homeostasis of the surface epithelium. Many of their target genes (like E-cadherin, desmoglein 1, PTEN, hTERT, PCNA and others) were previously implicated in carcinogenesis. Based on literature data and our preliminary results we hypothesized that reduced expression of the GRHL genes may increase susceptibility to epidermal carcinogenesis. Preliminary studies from our laboratory have shown that reduced Grhl1 expression in mice increases the incidence of DMBA/TPA-induced non-melanoma skin cancers (NMSC). It has also been demonstrated that Grhl3 knock-out mice are more susceptible to skin lesions upon chemical carcinogenesis. Links to oncogenesis for Grhl2 have been shown in in-vitro studies. In human skin cancers, reduced level of Grhl3 expression was observed in head and neck NMSC (by 90% in over half of the samples studied).

The aim of our research is to investigate whether various types of human skin cancers derived from epidermal cells are accompanied by changes in the expression levels of GRHL genes and to find out causes of these changes in a genetic and epigenetic context. We specifically search for: upregulation or downregulation of GRHLS expression, specific point mutations, loss of heterozygosity and copy number variation, changes of methylation profile in regulatory sequences, miRNAs specifically regulating GRHLS expression. Global changes in transcriptomes of different NMSCs with different GRHLS expression are also studied. To detect and identify GRHL gene disruptions in skin cancers, we use: New Generation Sequencing, DNA-methylation analysis, Human Gene Expression Microarrays, Lentiviral-based systems with miRNAs. Our findings will provide new molecular insights into the links between the GRHL genes and epidermal neoplasia in the human context.

SW04.S17–57
Mechanisms of coupling of the cancerogenesis processes and metabolism disorders under neoplastic transformation

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The aim of the paper was searching for coupling factors of metabolic disorders and malignant transformation processes in carcinogenesis. Blood plasma samples of 71 patients with malignant tumors of epithelial tissues and 31 healthy persons have been studied. Biochemical parameters of blood plasma have been estimated by using biochemical analyzers. Free radical activity has been assessed by induced biochemiluminescence technique, while oxidative modification of proteins has been determined by protein carbonyl derivatives level. An elemental analysis has been realized using the method of atomic emission spectrometry.

An albumin level significant decrease as well as urea content increase was detected in blood plasma. The main causes for such changes are supposed to be the protein catabolism processes and the increase of alfa-1 and gamma-globulins fractions induced by tumor stress response.

A significant increase of glucose level, as well as high levels of glycated hemoglobin and immunoreactive insulin, without any differences in C-peptide level indicate insulin resistance formation even at the initial stage of disease. The mechanisms of insulin resistance can be related to some disturbances of insulin receptors functioning under the influence of activated free radical oxidation and oxidative modification of proteins. Sodium level decrease (about 15%), potassium level (17%) and phosphorus level (20–58%) increase were found in blood plasma compared to the control group. Correlation analysis revealed a significant correlation between free radical activity and potassium content (r = −0.438) and sodium content (r = 0.488), which can be formed as a result of Na+/K+-ATPase oppression by reactive oxygen species (ROS). Trace Element Analysis showed a decrease in iron concentration (30–40%), copper (18–40%) and lithium (3–1 times) concentrations and increase of strontium content (90%). Lithium inhibits β3 glycogen synthase kinase, which is the main link of Wnt signaling pathway. The disturbance of Wnt signaling pathway can provoke malignant tumors formation. β3 glycogen synthase kinase regulates the activity of protein p53 responsible for cell cycle arrest or apoptosis. Alternatively, lithium has inhibiting effect on glucose-induced insulin release and inhibits lipid peroxidation.

Thus, mineral metabolism violation exactly is supposed to be the interlink between cells malignant transformation processes and metabolic disturbances in carcinogenesis.

SW04.S17–58
Regulation of hTERT transcription by the transcription factor KLF2 and DNA methylation in human T cells

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hTERT (human telomerase reverse transcriptase) is a catalytic subunit of telomerase that is strictly regulated in its expression in normal cells. Constitutive expression of hTERT is one of the common signatures of tumor cells, even though its promoter is mostly DNA-methylated. DNA methylation of promoters in
The role of Poly(C)-Binding Protein 2 in human gliomas growth

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Poly(C)-Binding Protein 2 (PCBP2) is a member of Poly(C)-binding protein family, which plays an important role in posttranscriptional and translational regulation by interacting with single-stranded poly(C) of target mRNAs. Several members have been reported to be involved in human malignant tumors. Although it is also discovered that PCBP2 functions in cancer progression, its role in glioma formation and development are yet to be elucidated. Here we show that PCBP2 is up-regulated in human glioma tissues and cell lines. Knockdown of PCBP2 by siRNA inhibits the growth of T98G, U87MG and U251 glioma cell lines through inhibiting of cell cycle progression and inducing of apoptosis. Moreover, an in vivo study confirmed that the tumor growth in nude mouse xenografts was significantly decreased in the Adv-PCBP2 siRNA group. We also identified several genes as target mRNAs binding to PCBP2 through RIP-Chip and biotin pull-down. Knockdown of PCBP2 enhances the expression of these genes through stabilizing their mRNAs. Our data suggest a potential character of PCBP2, which could be a novel biomarker and a therapeutic target to gliomas. Our data suggest a potential character of PCBP2, which could be a novel biomarker and a therapeutic target to gliomas.

SW04.S17–60
The association of TGFBR3 gene polymorphisms with endometrial cancer

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Introduction: Recent studies has indicated that deregulations of TGFβ signalling pathway play a crucial role in development and progression of different cancer types. Signal transduction in TGFβ cascade occurs via two types of signalling receptors, i.e., TGFβRI and TGFβRII, which are serine/threonine kinases. Signal induced by TGFβ ligands is modulated by accessory receptors deprived of enzymatic domain. Member of the accessory receptors family is betaglycan, encoded by TGFBR3 gene. Betaglycan is a transmembrane proteoglycan responsible for ligand presentation to its dedicated receptors. We have previously reported that betaglycan mRNA expression has been significantly downregulated with concomitant protein upregulation, in endometrial cancer comparing to normal endometrial mucosa [Zakrzewski et al. 2011]. Obtained results has become the basis for investigation of molecular alterations responsible for observed imbalance between transcriptomic and proteomic levels, particularly molecular mechanisms underlying observed transcriptome downregulation.

Aims and methods: The aim of the current study was to define the frequency of six single nucleotide polymorphisms (SNP) located in coding sequence (rs1805113, rs1805110, rs2296621) and promoter/regulator region (rs883873, rs2770186, rs12141128) in TGFBR3 gene and to estimate their associations with risk of endometrial cancer. The studied group consisted of 133 cases of endometrial cancer versus 248 healthy control patients. SNP genotyping analysis was performed by allelic discrimination using commercially available fluorescent TaqMan® probes.

Results: Our research indicated that the presence of CT and AG genotypes in the case of polymorphic sites rs2770186 and rs12141128 respectively, are correlated with higher occurrence of endometrial cancer (rs2770186 – OR 1.51, 95% CI 0.99–2.31, p = 0.05; rs12141128 – OR 1.51, 95% CI 0.99–2.32, p = 0.05).

Conclusion: In conclusion, we suggested that polymorphisms in the sequence located upstream of TGFBR3 gene (rs2770186 and rs12141128) seem to be associated with the increased risk of endometrial cancer.

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SW04.S17–61
SHP-1 regulates gene expression through changes in epigenetic modifications in prostate cancer cell lines

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SHP-1 is a phosphotyrosine phosphatase (PTP) preferentially expressed in hematopoietic cells. Our group demonstrated for the first time that this protein is also expressed in prostate and plays an important role regulating the cell cycle of prostate cancer cells. Since we have previously found that SHP-1 is present in both cytoplasm and nucleus, in this work we have considered the
possibility that SHP-1 could exert part of these effects by regulating gene expression through changes in epigenetic modifications. To this end we have compared the methylation levels of 1505 CpGs present in the promoter region of 807 genes in the PC3 and LNCaP prostate cancer cell lines depleted in SHP-1 by siRNA. Our data indicate that in LNCaP cells, that express high levels of SHP-1, the lack of SHP-1 expression decreases the methylation of 18 genes, while no significant changes where observed in PC3 cells, that express lower levels of SHP-1. Of the selected genes, the decrease in the methylation of GSTP-1, RUNX1T1, NPY, HIN-1 and KIT was correlated with an increase of the mRNA expression. Furthermore, the treatment of LNCaP cells with the demethylating agent 5-Aza-2deoxicitidine produced an increase in the mRNA expression of these genes similar to that observed with the SHP-1 depletion, while no changes at the mRNA levels of genes whose expression was not affected by SHP-1 ablation. In addition, the higher levels of RUNX1T1 and NPY mRNA observed in PC3 when compared to LNCaP cells were correlated with the unmethylation state of these genes in PC3 and with an increase in the histone marks related with gene expression (AcH3 and K4H3me3) in this cell line. In conclusion, our data suggest that in prostate cancer cell lines SHP-1 regulates gene expression by a mechanism that involves, at least in part, changes in epigenetic modifications.

**SW04.S17–62**

**Protein tyrosine phosphatase SHP-1 regulates prostate cancer cell migration**

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SHP-1, a tyrosine phosphatase with two SH2 domains widely expressed in haemopoietic system, is also a crucial phosphatase in epithelial cells. Our group demonstrated for the first time that this protein is expressed in prostate and play an important role regulating the cell cycle of prostate cancer cells. In this work, we have analyzed the possibility that SHP-1 play a role in the control of cell migration, a key event for tumour progression. We silenced SHP-1 expression in several cell lines derived from prostatic metastases and we assessed their migratory capacity through collagen or fibronectin. Our data indicate that SHP-1 ablation decreased the migration of the androgen-independent cell line PC-3 cell on collagen type I. In these cells, SHP-1 associates with FAK, Src and p85 subunit of PI3K, all of them signal molecules implicated in cell motility. SHP-1 ablation modified the composition of this complex leading to an increase in the phosphorylation and activity of src and a decrease of FAK phosphorylation and PI3K activity. This effect was cell-line dependent because SHP-1 ablation increased the migration of the androgen-sensitive cell line LNCaP. In summary, SHP-1 regulates prostate cancer cell migration by interaction with FAK, PI3K and src in a signaling platform.

**SW04.S17–63**

**Role of Opisthorchis felineus in the induction of bile duct cancer in experimental opisthorchiasis**

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Opisthorchiasis is food borne trematodiasis, the causative agents of which are liver flukes – Opisthorchis felineus and O. viverrini (Opisthorchiidae, Trematoda). O. felineus is epidemiologically significant on the territory of former USSR. O. viverrini is widespread in South East Asia. According to different estimations, up to 40 million people are currently infected with these liver flukes and up to 600–750 million people in Eurasian countries constitute the risk group. It has been shown O. viverrini induces cholangiocarcinoma in hamsters treated by nitrosamines. Carcinogenic characteristics of O. felineus are still unknown.

Purpose of this work was the study of O. felineus carcinogenic characteristics in hamsters treated by dimethylnitrosamine (DMN). One hundred seventy golden Syrian hamster were divided into four groups: (I) untreated control, (II) treated with 12.5 ppm DMN solution alone, (III) infected with 50 metacercaiae of O. felineus alone and (IV) infected with 50 metacercaiae of O. felineus and treated with 12.5 ppm DMN solution. Four or six hamsters from each group were sacrificed every 4 weeks. Livers of hamster were subjected to routine histological processing.

No pathological changes were found in group I. Examination of group II revealed periportal lymphocytic infiltrations, increasing dysplasia and nodular hyperplasia of hepatocytes. Group III showed lymphocytic infiltrations, periductal fibrosis, bile ducts goblet cell metaplasia and hyperplasia, cholangiolibrosis and adenomatous polyps. Pathological changes in group IV were more severity and combined features of groups II and III. Furthermore, cholangiocarcinoma was found at 18 week.

Precancerous lesions (group III) and cholangiocarcinoma (group IV) indicate that O. felineus plays a crucial role in the induction of bile duct cancer in hamsters model of opisthorchiasis.

**SW04.S17–64**

**Genetic differences in nuclear receptors Car and AhR activation and expression in different mouse strains after o-aminoazotoluene application**

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Over the last few decades the key role of some nuclear receptors (NR) in liver tumorigenesis was established. Constitutive androstan-3-one receptor (Car, Nr1l3) activation was shown to promote liver tumor development (1). Aryl hydrocarbon receptor (AhR) activation also cause tumors in liver, but this receptor can also act as tumor suppressor in certain conditions (2). Inbred mouse strains differs in susceptibility to carcinogenesis are an appropriate model for molecular mechanisms of carcinogenesis investigation.

In this work o-aminoazotoluene (OAT) effect on hepatic nuclear receptors AhR, Car and their target genes Cyp1a1 and Cyp2b10 expressions of susceptible (DD/He) and resistant to chemical carcinogenesis (CC57BR/Mv) mice strains was studied. We examined Cyp1a1 expression as a marker of AhR activation
and Cyp2b10 induction for CAR activation. Quantitative real-time PCR was used for gene expression estimation. It was demonstrated that the basal AhR, Cyp1a1 and Cyp2b10 genes expression in hepatic tissue of DD/He (DD) and CC57BR/Mv (BR) mice did not differ, whereas relative content of Car mRNA was reliably higher in resistant strain. Chronic OAT application caused an increased expression of both target genes, but BR strain demonstrated more prolonged and considerable increase in Cyp1a1 expression, whereas DD strain showed more enhanced level of Cyp2b10 expression. The predominant activation that was suggested for AhR signaling pathway within resistant BR strain and for Car-mediated pathway within susceptible DD strain.

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References

SW04.S17–65
Regulation of apoptosome apparatus in non-small cell lung cancer cells and tissues
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Aim: The apoptosome apparatus (AA) is an important platform of mitochondrial cell death signaling. Evasion or insufficient induction and course of AA signaling may contribute to tumourigenesis, progression and therapy resistance of neoplasms. To comprehend the functionality of AA in non-small cell lung carcinoma (NSCLC), we investigated its activatability and regulation in NSCLC cells and tumours and matched lungs.

Methods: The activatability of AA in cell-free cytosols was measured upon addition of cytochrome-c (cyt-c) and dATP as the induced caspase (CS)-3-like activity using the fluorogenic tetrapeptide substrate Ac-DEVD-AFC. Cytosols were prepared from 62 NSCLC tumours and matched lung parenchyma obtained from surgically treated patients and from 7 NSCLC cell lines.

Results: The addition of cyt-c and dATP induced more than twofold increase of CS-3-like activity in 19 of 62 cytosols from NSCLC tumours and in five of 62 cytosols from the lungs. The endogenous as well as the (cyt-c + dATP)-induced CS-3-like activities were significantly higher in NSCLC tumours as compared to the lungs. However, XIAP-neutralizing peptides AVPIAQK and ATPFQEG were unable to revert the resistance to the (cyt-c+dATP)-mediated AA activation in the tumour cytosols. Gel filtration chromatography and Western blot analysis showed that in the (cyt-c+dATP)-treated A549 cell cytosol, the apoptosome components Apaf-1 and procaspase-9 (PC-9) eluted in the low- as well as high-Mr fractions, indicating a high-Mr apoptosome complex formation without PC-9 processing. In addition, the major quantity of PC-9 was present in low-Mr fractions. On the contrary, in the (cyt-c+dATP)-treated cytosol samples from COLO-699 and CALU-1 cells, all PC-9 was converted to CS-9 and eluted together with Apaf-1 in the high- as well as low-Mr fractions.

Conclusions: The results of the present work indicate that a large subset of NSCLC tumours and lungs are resistant to the (cyt-c+dATP)-mediated AA activation and that XIAP is not the major suppressor of AA signaling in NSCLC. In A549 cell-free cytosol, the failure of apoptosome-bound PC-9 activation/activity may contribute to AA dysfunction.

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SW04.S17–66
Expression of Nogo-A and Nogo-A/B in invasive ductal breast carcinoma and non-small cell lung cancer
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Nogo proteins (Nogo-A and Nogo-B) are relatively newly discovered proteins with many potential unidentified functions. Nogo-A expression is restricted mainly to the nervous system, whereas Nogo-B expression is expressed in various tissues. Recent studies indicated that Nogo-B may act as an oncosuppressor protein as its decreased expression was observed in small cell lung cancer and human adult T-cell leukemia. Moreover, depending on cell type, Nogo-B was shown to impact cancer cells migration and susceptibility to apoptosis in vitro.

In this study, we investigated Nogo-A and Nogo-A/B in 233 invasive ductal breast carcinomas (IDC) and 130 non-small cell lung cancers (NSCLC) in paraffin sections by immunohistochemical (IHC) methods using primary goat polyclonal antibodies. The intensity of Nogo-A and Nogo-A/B expression was analyzed under light microscope utilizing the semiquantitative assessment scale based on the percentage of positive cells and the reaction intensity.

Analysis of IHC sections revealed no or weak cytoplasmic Nogo-A expression in both tumour types, whereas a cytoplasmatic-membrane Nogo-A/B expression was noted in all the analyzed IDC and NSCLC cases. Statistical analysis showed, that low Nogo-A/B expression in IDC cancer cells was associated with larger primary tumour size ($p = 0.049$) and the triple negative phenotype ($p = 0.03$). No associations of Nogo-A/B expression and lymph node metastases, patients age, menopausal status and expression of estrogen, progesterone and HER-2 were noted. In NSCLC a significantly lower Nogo-A/B expression was noted in adenocarcinomas as compared to squamocellular carcinomas ($p < 0.0001$). However, in NSCLC Nogo-A/B expression had no impact on primary tumour size, presence of lymph node metastases, patients age and sex. Survival analysis revealed that IHC expression of Nogo-A and Nogo-A/B yielded no prognostic significance in IDC, as well as NSCLC.

In conclusion, Nogo-B seems to be the most expressed Nogo isofrom in comparison to absence of Nogo-A expression. However, the results do not allow to unanimously define Nogo-B as an oncosuppressor.
SW04.S17–67
Regulation of Par-4 rat versus human by casein kinase 2 in prostate cancer cells
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Prostate apoptosis response-4 (Par-4) has a strong anti-oncogenic potential in prostate cancer cells, via its pro-apoptotic functions. Casein kinase 2 (CK2) is a key protein in prostate cancer resistance, but how CK2 supports survival is poorly known. Our findings reveal a differential inhibitory role of CK2 on Par-4 functions in human versus rodent by inducing its phosphorylation on serine residues S124 and S223 in rodent and S231 in human (ortholog of rodent S223). In rodents, S124 phosphorylation prevents the caspase-mediated generation of the fully active cleaved form of Par-4. Indeed, once cleaved, Par-4 is secreted and endows all pro-apoptotic functions of Par-4 full-length. In human, by means of phosphohmutants, we show that CK2 phosphorylates Par-4 on S231 (S223 ortholog of rat Par-4) and, consequently, impairs apoptosis. Furthermore, Par-4 phosphorylation at S231 is specifically detected in human prostate cancer cells, which display elevated CK2 activity, but not in their normal counterpart. Finally, sensitization of prostate cancer cells to apoptosis by CK2 knockdown is significantly reversed by Par-4 siRNA. Thus, we define a pro-tumorigenic role for CK2 through Par-4 inhibition.

SW04.S17–68
Role of WT1-ZNF224 interaction in the expression of the tumor suppressor interferon regulatory factor 8 in leukemia cells
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Wilms’ tumour 1 protein, WT1, is a zinc finger transcriptional factor that exerts an oncogenic role in a variety of tumours from different origins, including leukemias. The WT1 transcriptional isoforms more extensively studied are WT1(+/−) and WT1(+/+), resulting from the use or skipping of exon 5.

The transcriptional regulatory properties of WT1 are influenced by interaction with different molecular partners. Recently, we identified the Krüppel-like zinc finger protein, ZNF224, as a novel human WT1-associated protein and demonstrated that ZNF224 may act as a transcriptional co-regulator of WT1(+/−) to modulate the expression of apoptosis regulating genes [1, 2].

The Interferon regulatory factor 8 (IRF8), known as a tumor suppressor in some leukemias, is a novel WT1 target gene. WT1(+/−) isoform negatively regulates IRF8 gene expression through a transcriptional mechanism [3]. In the present study, by immunoprecipitation assay we demonstrated the interaction between ZNF224 and WT1(+−-KTS) and identified the critical domains involved in this interaction. Furthermore, by chromatin immunoprecipitation and transient transfection experiments in CML K562 cell line we demonstrated that WT1(+−) recruits ZNF224 on the promoter region of IRF8 and that ZNF224 prevent WT1 (+−) to repress the IRF8 promoter transcription. The expression levels of IRF8 and ZNF224 are reduced or absent in most myeloid leukemia cell lines, when WT1 is overexpressed [3]. Furthermore, we observed that the induction of ZNF224 by ara-C or imatinib in K562 cells is accompanied by a significant increase of IRF8 expression.

Our data provide novel insights into the pathogenesis of leukemia with potential therapeutic implications.

References

SW04.S17–69
Effect of vascular endothelial growth factor (VEGF) on ADAMTS1 gene expression in hepatoma cells
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Vascular endothelial growth factor A (VEGF-A) is the most important mediator of angiogenesis and is overexpressed by a multitude of solid human tumors. VEGF-A increases microvascular permeability and promotes survival, migration, and proliferation of endothelial cells. A disintegrin and metalloproteinase with thrombospondin motif type 1 (ADAMTS1) is a recently discovered metalloproteinase with antiangiogenic activity. ADAMTS1 dysregulation is linked to the most commonly diagnosed cancers. But, conflicting reports currently surround its expression in cancer, as different studies have shown both up- and downregulated expression of ADAMTS1 in primary tumors compared with healthy tissue controls. For this reason, ADAMTS1 has been ascribed both pro- and anti-tumorigenic activities but with poor understanding of the specific mechanisms it mediates to promote or inhibit tumorigenesis.

The aim of the work is to evaluate the effect of VEGF on the expression of the ADAMTS1 and VEGF mRNA by qRT-PCR in Hep3B cells. The gene expression levels of these genes were investigated under normoxic and hypoxic conditions in Hep3B (Human hepatoma cell line) with serum free condition including 0.1% BSA. Chemical induced hypoxic conditions in Hep3B cells were created with 150 µM final concentration of CoCl. In addition, the expression of HIF1α (Hypoxia Inducible Factor-1a) which is the indicator of hypoxic conditions were analyzed. Hypoxia conditions were correlated with upregulation of the transcription factor Hypoxia Inducible Factor-1a mRNA levels. Hep3B was treated different concentration of VEGF in different time intervals, 1, 3, 6, 24 and 48 hours. Expression levels of ADAMTS1 were upregulated in hypoxic condition. In addition, VEGF upregulates the ADAMTS1 and VEGF mRNA level at early time points. Moreover, upon the incubation of VEGF, immunofluorescence analysis of ADAMTS1 was carried out at protein level and compared to untreated control group.
SW04.S17–70
Superoxide-dependent uptake of vitamin C in human glioma cells
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Glioblastomas are lethal brain tumors that resist current cytostatic therapies. Vitamin C may antagonize the effects of reactive oxygen species (ROS) generating therapies; however, it is often used to reduce therapy-related side effects despite its effects on therapy or tumor growth. Because the mechanisms of vitamin C uptake in gliomas are currently unknown, we evaluated the expression of the sodium-vitamin C cotransporter (SVCT) and facilitative hexose transporter (GLUT) families in human glioma cells. Additionally, as microglial cells can greatly infiltrate high-grade gliomas (constituting up to 45% of cells in glioblastomas), the effect of TC620 glioma cell interactions with microglial-like HL60 cells on vitamin C uptake (Bystander effect) was determined. Although glioma cells expressed high levels of the SVCT isoform-2 (SVCT2), low functional activity, intracellular localization of the dominant-negative isoform (dnSVCT2) was observed. The increased glucose metabolic activity of glioma cells allowed high 2-Deoxy-D-glucose (2-DOG) and dehydroascorbic acid (DHA) uptake rates through the GLUT isoform-1 (GLUT1), the main DHA transporter in glioblastoma. Co-culture of glioma cells and activated microglial-like HL60 cells resulted in extracellular ascorbic acid (AA) oxidation and high DHA uptake by glioma cells. This Bystander effect may explain the high antioxidative potential observed in high-grade gliomas.

SW04.S17–71
RAG1/2 recombinase introduces lesions at cryptic recombination signal sequences that drive lymphomagenesis
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Lymphoid neoplasms continue to be the main cause of cancer in children and many adults. RAG1/2 recombinase, essential for lymphocyte development, is speculated to be involved in generation of this disease due to its intrinsic endonuclease activity. In this study, using the p53-deficient RAG2 ‘core’ mouse model of lymphoma that unmasks the aberrant nuclease activity, we present the first comprehensive analysis of the RAG1/2 impact on genomic integrity. We show that RAG1/2 introduces multiple rearrangements through aberrant recombination outside of the V(D)J loci, many of them bearing tumorigenic potential. We perform detailed analysis of cryptic recombination signal sequences driving the aberrant RAG1/2 activity and find sequence requirements that will help elucidate the potential impact of RAG1/2 on the human genome as well as make possible to identify genetic variants with predisposition to RAG1/2-mediated genomic instability. The analysis of recurrent RAG1/2-induced mutations in this mouse model offers multiple clues leading not only to identification of genes/pathways involved, but also gives insight into the spectrum of oncogene-activating rearrangements in the human disease.

SW04.S18 Mechanisms of G Protein Signaling (IV-S18)

SW04.S18–1
Rap signalling complexes: landmark recognizing modules in cell adhesion and polarity
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Rap proteins are Ras-like small GTPases that regulate cell adhesion and cell-cell junction formation. Rap1 proteins are spatially and temporally regulated by a variety of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins. These proteins are thought to recognize specific landmark proteins to translate spatial cues into action. For Epacl, a cAMP-regulated GEF for Rap proteins, a number of membrane anchors have been identified that localizes Epacl at different positions in the cell. For instance, we recently found that Epacl1, through its DEP domain, tethers to phosphatidic acid at the plasma membrane, an interaction that is regulated by cAMP (Consonni et al., 2012 PNAS 109, 3814).

Downstream from Epacl and Rap1 we identified a novel effector for Rap1, Radil, an adaptor protein that interact with ArhGAP29 to inhibit Rho, both in cell spreading and endothelial barrier function (Post et al., submitted).

Finally, in intestinal brush border formation, apical PI4, 5P2 activates phospholipase D to induce apical phosphatidic acid, which serves also as a landmark for another RapGEF, PDZ-GEF. The resulting localized activation of Rap2A is translated into brushborder formation though the Rap2 effector, the serine/threonine kinase TNIK, the serine/threonine Mst4 and phosphorylation of Ezrin (Gloerich et al., 2012 Nature Cell Biol 14, 793).

SW04.S18–2
Catalysis of small GTPases by their respective GAP's
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The catalytic mechanism of several small GTPases by their respective GAPs is studied spatio-temporal resolved in great detail. Time-resolved FTIR difference spectroscopy is used to monitor protein reactions and interactions at atomic detail with ms time-resolution [1]. This provides in combination with structural models and biomolecular simulations (Molecular Dynamic and QM/MM) also spatial resolution [2]. Catalysis of several small GTPases, Ras, Ran, Rap, Rho and Rab is studied. The dynamics of the catalytic site will be discussed [3,4,5], especially of Rab. The binding of GTP to Ras and GAPRas drives it into a eclipsed configuration as compared to staggered in water and thereby closer to the transition state confirmation [6]. This reduces the free activation energy and explains beside the common stabilisation of the attacking water molecules by an amide the protein catalysis by 10 orders of magnitude.

In order to investigate in addition the lipid bound Ras and its interaction network the ATR (attenuated total reflection) technique is applied [7,8]. Surprisingly a dimerization of the membrane anchored Ras in POPC membranes is identified and confirmed by FRET measurements and MD simulations [9].
called downstream effectors. These are the delta subunit of phosphodiesterase and similar proteins. Compartmentalization in the cell is mediated by factors such as PDE isoforms.

- Ras proteins rely on a highly dynamic shuttling between different membrane compartments. Transport between membrane attachment and function. Spatiotemporal regulation of Ras proteins is critical for the activation of downstream effectors.

- The delta subunit of phosphodiesterase and similar proteins are regulated by Guanine nucleotide exchange factors (GEFs) that switch Ras proteins between a GDP-bound OFF and a GTP-bound ON state.

- Ras itself and RheB have a C-terminal CaaX motif, the cysteine residue of which is modified with the lipophilic farnesyl moiety. Farnesylation of Ras proteins is catalyzed by farnesyl transferase, a post-translational modification.

- Over 150 human proteins comprise C-terminal CAAX motifs, including Ras and RheB. Modification of the CAAX motif through a cascade of reactions is essential for targeting these proteins to the plasma membrane or other subcellular compartments.

We will discuss how spatiotemporal regulation of Ras proteins is mediated by the Arl2 and 3 conformational switch cycle and how this has suggested a new avenue towards anti-Ras drugs.

**SW04.S18-4**

**Structure and mechanism of the CAAX motif processing enzymes ICMT and RCE1**

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Over 150 human proteins comprise C-terminal CAAX motifs, including Ras proteins. Modification of the CAAX motif through a cascade of reactions is essential for targeting these proteins to the plasma membrane or other subcellular compartments.

- Ras signalling from the plasma membrane is dependent on post-translational modification of the CAAX motif. These events involve prenylation (farnesylation) of the CAAX motif by the farnesyl transferase. Endoproteolysis of the CAAX motif liberates the aX tripeptide.

**SW04.S18-5**

**Cdc42-based mechanism of cell fate differentiation in budding yeast**

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Budding yeast is an ideal model organism for studying the principles underlying eukaryotic cellular morphogenesis. Rather than dividing its cell in halves, this unicellular fungus proliferates by constructing every new daughter cell de-novo, on the side of the mother cell. This process is an amazing example of building a cell from scratch. It starts with the establishment of a new cell polarity axis, which is physically marked by a membrane domain, prescriptive bud site (PBS), whose protein-lipid composition is distinctly different from the rest of the cell. This and the whole cascade of downstream morphogenetic processes are controlled by a single master regulator – small GTPase Cdc42. Its biological activity is directly responsible for the assembly of the PBS and the following formation of the septin ring, a dense polymeric organelle that serves as the boundary between mother and daughter until they are finally split apart by cytokinesis. Septin rings are found at the cytoplasmic sites of fungi, in the tails of spermatozoa as well as at the base of neural spines and eukaryotic cilia – all places where contiguous membrane has to be divided into two non-mixing domains with distinct biological properties and developmental fates. Yet, despite the utmost importance of this organelle for eukaryotic cells, molecular mechanisms responsible for the formation of these rings are not known in any system. As often before, budding yeast comes to the rescue again. In this talk I will present the results of our recent experiment-theory study aimed to unravel the mystery of septin ring emergence in budding yeast. Among others, I will answer a long-standing question: How does it become a ring in the first place?
Targeting Ras proteins in human cancer
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Oncogenic Ras proteins play a major role in human cancer. They are thought as drivers of the majority of pancreatic cancers, and many cancers of the colon, lung, and other sites. Wild-type Ras proteins also play an important role in cancer, when negative regulators such as the NF1 protein, neurofibromin, are mutated or deleted. Ras proteins, in their GTP-state, bind and activate several potential effectors, including Raf kinases, PI3’kinase, and RalGDS. The precise roles of these effectors in cancer initiation, progression and maintenance remains unclear. Attempts to block these effectors have been largely unsuccessful. Raf kinase inhibitors paradoxically activate Raf kinase in Ras-mutant cancers, and MEK inhibitors provoke activation of upstream pathways through loss of negative feedback. Mechanisms for these effects will be presented and discussed.

To explore new ways of targeting Ras, we investigated differences between cells transformed by KRAS and by HRAS. KRAS is by far the major contributor to human cancer, whereas HRAS is rarely activated. In spite of this dramatic difference, KRAS and HRAS interact with the same effectors and are equally potent at transforming cells in culture. However, cells transformed by KRAS have unique properties relative to HRAS: they cause a stem-like phenotype that enables them to grow as spheres formed by KRAS have unique properties relative to HRAS: they cause a stem-like phenotype that enables them to grow as spheres. These effects appear to be due to KRAS’ ability to bind calmodulin, and so to inhibit calmodulin-dependent kinase. Low CaM kinase promotes wt signaling and initiates a set of programs that confer stemness. Therapeutic opportunities based on these discoveries will be presented.

The RA and PH domains of RIAM act as a proximity detector for Rap1 and P(4,5)P₂
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Among the primary modes of action of small GTPases is the recruitment of effectors to cellular membranes. One such small GTPase effector pair is Rap1 and Rap1 Interacting Adapter Molecule (RIAM). RIAM binds to talin and regulates outside-in signaling to integrins and thereby controls the adhesiveness of hematopoietic cells. Rap1 is distributed between the plasma membrane (PM), and endomembranes. Nevertheless, the association of RIAM with activated Rap1 was restricted to the PM, which is the compartment upon which integrins are regulated, raising the question of how spatial specificity is achieved. We show that RIAM is recruited to the lymphocyte PM through its Ras association (RA) and pleckstrin homology (PH) domains, both of which were required for lymphocyte adhesion. The N-terminus of RIAM inhibited membrane translocation. In vitro, the RA domain bound both Rap1 and H-Ras with equal but relatively low affinity, whereas in vivo only Rap1 was required for PM association. The PH domain bound phosphorylitol 4,5-bis-phosphate [P(4,5)P₂] and was responsible for the spatial distribution of RIAM only at the PM of activated T cells. We determined the crystal structure of the RA and PH domains and found that, despite an intervening linker of 50 amino acids, the two domains were integrated into a single structural unit, which was critical for proper localization to the PM. Thus, the RA-PH domains of RIAM function as a proximity detector for activated Rap1 and P(4,5)P₂. This establishes a paradigm in which two low affinity membrane binding domains combine to stabilize membrane association and wherein one of them, the PH domain, specifies the subcellular compartment upon which an effector engages a small GTPase.

Towards structural studies of ligand-induced conformational changes in arginine-vasopressin V2 receptor
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Human arginine-vasopressin V2 receptor (V2R) is primarily expressed in kidney tubules and is involved in mechanisms that concentrate urine and keep water homeostasis. Several biased ligands stabilize distinct conformational states of V2R leading to biased signaling pathways [1]. In order to elucidate conformational changes in V2R induced by ligand binding, we devised a research strategy based on conformational phi-value analysis. This protein-engineering method was originally applied to obtain structural information about transition states during protein folding [2]. In its modified form and combined with alanine scanning mutagenesis, conformational phi-value analysis becomes a valuable tool in the study of ligand-induced conformational changes in GPCRs. For this purpose, we measure thermal stabilities of each alanine mutant in presence and absence of a particular ligand. By comparing thermal stabilities of the mutants with those for the wild type, we can quantify the ligand-induced structural changes at a single-residue level. In addition, thermostability data could be used to engineer conformationally stabilized GPCRs [3] that are better suited for crystallographic and NMR studies. In designing V2R constructs for crystallographic studies, we are also investigating applicability of transferring some of the thermostabilizing point mutations and truncations from other GPCRs as well as replacing the intracellular loop 3 with different fusion partners [4].

Local alteration of protein-lipid interactions regulates bleb-driven chemotaxis in Dictostylium cells
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Migrating cells can adapt their mode of movement to the mechanical properties of their surroundings. We find that Dictyostelium cells use both actin-driven pseudopods and blebs for propulsion when moving under buffer, but switch to blebs for movement in a mechanically resistive environment. In comparison to pseudopods, blebs are projected 2–3 times faster, but are less persistent; they have little F-actin and leave behind an F-actin scar. Blebs are repopulated with F-actin within a few seconds of formation, and often give rise to pseudopods, making
them difficult to distinguish in standard chemotaxis movies, taken at low frame rates.

Blebbing cells are chemotactic to cyclic-AMP, with blebs predominantly forming at the front of the cell. In re-orientation experiments, where cells are induced to change direction by moving a needle releasing cyclic-AMP, blebs re-polarize to the new direction of travel within 30 s. Thus the site of bleb formation is under immediate chemotactic control, and does not depend on prolonged polarization of the cell.

A genetic screen shows that blebbing is abolished, or severely reduced, by mutation of the myosin-II heavy or light chains or upstream regulators of myosin. Conversely, blebbing increases in mutants impairing actin polymerization, such as in the SCAR complex, or if cortical function is impaired.

We have identified a signalling module that controls chemotactic blebbing in response to cyclic-AMP. It involves PI3-kinase and two downstream PI3-binding proteins, CRAC and PhdA. We have shown that PI3-kinases can locally regulate cell membrane blebbing in response to chemotactic stimulations. PI3-kinases transform PI(4,5)P2 lipid in the plasma membrane into PI(3,4,5)P3, consequently altering the interaction between plasma membrane and underlying cytoskeleton, as well as recruiting the effector proteins containing specific PH-domains – such as PhdA, CARC and PKB. Blebbing is severely impaired in a quintuple PI3-kinase mutant, or a double CRAC/PhdA mutant.

We conclude that Dictyostelium cells have two modes of propulsion – blebs and F-actin driven pseudopods – under separable biochemical control and favour blebs when they meet physical resistance. We suggest that blebbing movement may also be a response of metazoan cells to physical resistance, such as they resist. We suggest that blebbing movement may also be a biochemical control and favour blebs when they meet physical–pulsion

SW04.S18–10

Atypical Rho family GTPase Chp/RhoV induces apoptosis of PC12 cells

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Rho GTPases regulate numerous cellular processes including apoptosis. Chp/RhoV is an atypical Rho GTPase which functions are poorly understood. Chp is expressed early in embryonic development of chick, frog and zebrafish, being one of the earliest expressed neural crest markers in X. laevis embryos and playing an important role in differentiation of neural crest cells. In mammals RHOV transcript encoding Chp is found both in fetal and adult brain, but functions of Chp in the nervous system of mammals remain to be elucidated. JNK-dependent apoptosis is important for the proper development of the nervous system in many organisms including mammals and Chp was shown to stimulate JNK in HEK293 cells upon transient expression. PC12 rat pheochromocytoma cell line is often used as a model of neuronal differentiation, and activation of JNK pathway is crucial for apoptosis in these cells.

Here we investigated the role of Chp in regulation of cell viability using PC12 cells with inducible expression of Chp as a model. We found that expression of Chp results in apoptosis in PC12 cells. Chp-induced apoptosis was accompanied by activation of JNK signaling and both death receptor-mediated and mitochondrial apoptotic pathways as justified by caspase-8 and caspase-9 activation, respectively. Moreover, inhibition of JNK by SP600125 rescued PC12 cells from Chp-triggered cell death and attenuated activation of caspases-9 and -3, suggesting that activation of JNK mediates pro-apoptotic effect of Chp. Expression of Chp resulted in increased phosphorylation of c-Jun in PC12 cells, and Chp expression in HEK293 cells up-regulated AP-1-dependent transcription in a JNK-dependent manner. Together results of our study reveal the role of Chp GTPase as a putative regulator of JNK-dependent apoptotic death in PC12 cells, similarly to previously described pro-apoptotic activity of the related Cdc42 and Rac1 GTPases.

SW04.S18–11

Myocardial beta-adrenergic signaling in spontaneously hypertensive rat: the effect of transgenic rescue of defective Cd36 gene

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Spontaneously hypersensitive rats (SHR) harbor a deletion variant of Cd36 gene that results in reduced transport of long chain fatty acids. There are some indications that that β-adrenergic responsiveness is attenuated in different tissues of SHR when compared to control rats. Here we investigated the key elements of β-adrenergic signaling in the heart of SHR of transgenic strain Tg19 (SHR-Tg19 or SHR-Tg36) and in the corresponding SHR controls. Expression and distribution of β1- and β2-adrenergic receptors (β-ARs) in samples of heart tissue were measured using radioligand binding and Western blot analysis. Expression of selected G proteins was also assessed. In parallel, activity of adenyl cyclase (AC) was determined. There were no significant changes in the expression of Gsa and Gia subunits in both the left (LV) and right (RV) ventricles. Radioligand binding revealed an increase in the total amount of myocardial β-ARs, which was apparently brought about by increased expression of β2-ARs. The expression of AC type 5/6 was increased in the LV of SHR-Tg19 and the AC activity stimulated by different stimulators was also significantly higher in heart tissue samples of transgenic strain as compared to SHR controls. Overall, transgenic rescue of Cd36 gene in SHR seems to enhance the function of myocardial β-adrenergic signaling pathway.

SW04.S18–12

P2Y6-induced release of ATP from the urothelium exerts a dual role in the human urinary bladder

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Bladder distension, inflammation and chemical irritation stimulate the release of nucleotides leading to bladder overactivity. ATP-sensitive P2X1 receptors prevail in the detrusor smooth muscle, whereas P2X3 receptors found in pelvic nerve afferents trigger the micturition reflex upon bladder filling. In contrast to the compelling evidence for the signaling role of extracellular ATP, we now investigated if uracil nucleotides play a role in the human urinary bladder collected from cadaveric organ donors. Human urothelium exhibits significant UDP-sensitive P2Y6 immunoreactivity. The selective P2Y6 receptor agonist, PSB0474 (100 nM), increased the release of ATP and [3H]ACh respectively by 50 ± 12% (n = 3) and 21 ± 6% (n = 3) from the human urothelium, an effect that was antagonized by the P2Y6 antagonist, MRS2578 (50 nM). The effect of PSB0474 (100 nM) on bladder urothelium contrast with the inhibitory (~50%) response observed on [3H]ACh release from...
stimulated cholinergic nerves innervating the detrusor; the P2Y1 antagonist, MRS2179 (0.3 μM), prevented inhibition produced by PSB0474 (100 nM) on evoked [3H]-ACh release. Data suggest that activation of P2Y1 receptors operates a dual role in the human urinary bladder by releasing ATP from the urothelium. While ATP operates bladder overactivity via P2X3 and P2X1 receptors located respectively on suburothelial sensory nerves and smooth muscle fibers, bladder excitation may be partially counteracted by ADP generated from the catabolism of ATP by ecto-NTPDases via the activation of inhibitory P2Y1 receptors on cholinergic nerves. In the human urinary bladder ADP may generate via ecto-NTPDases 2 and 3 expressed in urothelial and suburothelial layers as detected by confocal microscopy. Work supported by FCT (PTDC/SAU-OSM/104369/2008 and Pest-OE/SAU/UI215/2011), APU and UP/CGD.

**SW04.S18-13**

Chemokine receptor antagonists influence on monocytes infiltration through endothelial cells monolayer studied by a real-time electrical impedance assay

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**Background and aims:** Atherosclerosis is characterized by an inflammatory process and a specific repertoire of chemokines and cell adhesion molecules expressed by endothelial cells (EC) that attract leukocytes to the developing atheroma. The inhibition of chemokine/chemokine receptor interactions and prevention of accumulation of pro-inflammatory cells in the vascular wall may constitute an important therapeutic option. Our aim was to follow in real-time the effect of chemokine receptor antagonists (CRA) on monocyte adhesion and transmigration through endothelial cell monolayer.

**Materials and Methods:** We used a method based on changes in electrical impedance to study in real-time the monocytes infiltration through endothelial cell monolayer. The endothelial cells (line EA.hy926) were cultured in 16-well e-plates covered with gold microelectrode that monitors and quantifies the infiltration of human monocytes (U937 cell line) in the presence/absence of CRA specific to chemokine receptors CCR2 (Tejini1 compound 1 and BMS CCR2) and CCR5 (maraviroc) and a broad-range CRA (pertussis toxin).

**Results:** The formation of EC monolayer was documented by the increase in electrical impedance in the first 24 hours of the experiments. The infiltration of monocytes, characterized by the opening of endothelial cell junctions, retraction of endothelial monolayer and replacement by monocytes determined a significant decrease in impedance. In contrast, the incubation of EC with monocytes in the presence of chemokine receptors antagonists lead to increases in impedance values indicating that monocytes infiltration was inhibited in the presence of CRA. The percentage of increases in impedance were calculated at different time points over 3 days and the end of experiment the values were as follows: for Tejini compound 1 by ~17%, for BMS CCR2 and maraviroc by ~30% and for PTX by ~20%.

**Conclusion:** This novel technique monitors in real-time the effect of CRA on adhesion and transmigration of monocytes through endothelial monolayer. The advantages of this method in comparison with other methods (Boyden chamber, matrigel assay) are: (i) the endothelial cell-monocyte interaction mimics more closely the in vivo process, and (ii) the data are obtained in real-time and are more reliable quantify as opposed to end-point analysis for other methods.

**Acknowledgements:** This work was supported by Romanian Academy, UEFISCDI, contract no. 4_001 under the frame of EuroNanoMed and CARDIOPRO Project ID: 143, ERDF co-financed investment in RTDI for Competitiveness. The financial support from European Social Fund ID POSDRU/107/1.5/S/ 82839 (V.S.) are gratefully acknowledged.

**SW04.S18-14**

The Intersectin-1L splice variant delta 35 is capable to activate Cdc42 but also binds RhoU

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ITSN1 is a highly conservative multidomain scaffold protein implicated in membrane trafficking and cell signaling. It has two main isoforms. The short one (ITSN1-S) is ubiquitously expressed and consists of two N-terminal Ep15 homology (EH) domains, coiled-coil region (CCR) and five SH3 domains. The long isoform (ITSN1-L) is expressed predominantly in neurons and possesses additional C-terminal dbl homology (DH) and pleckstrin homology (PH) followed by C2 domain [1]. The DH domain is known to be a specific guanine nucleotide exchange factor (GEF) for Cdc42, a member of the Rho GTPase family [2].

In addition to the abovementioned major signaling events, lots of minor alternative splicing events have been reported for ITSN1. They give rise to the set of proteins with modified size and domain composition [3]. Although significant progress has been made toward understanding ITSN1-S and ITSN1-L functions, the role of its minor isoforms in different cellular processes remains unclear.

We have recently described an isoform which lacks exon 35 and thus, encodes a protein with a modified GEF-domain (ITSN1-Ldelta35). This minor isoform is widely expressed along with the entire ITSN1-L [4]. The excision of exon 35 leads to the loss of α6 helix of DH domain which results in shortened distance between DH and PH domains. This may lead to the contact of PH domain with the target GTPase which normally does not happen with entire DH-PH domains. We have assumed that the latter may result in the change in affinity towards existing partner Cdc42 as well as to the change of specificity.

We have demonstrated that ITSN1-Ldelta35 DH-PH domains still bind Cdc42 and are capable to activate it as well as the wild type DH-PH domains. Besides, we have also discovered a new specific partner of ITSN1-Ldelta35 DH-PH domains – RhoU (Wrch1), an atypical small Rho GTPase, which is closely related to Cdc42. But whether ITSN1-Ldelta35 is a GEF for RhoU and what is their common function remains to be investigated.

**References**


SW04.S18–15
Involvement of EGFR signaling in colon cancer cell migration
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Cell migration plays an important role in metastasis. A better understanding of the molecular mechanisms of cell locomotion is therefore of high clinical relevance.

Epidermal growth factor (EGF) is a well-known growth factor that induces formation of dynamic cell protrusions associated with the actin cytoskeleton remodeling and cell migration [1]. Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase which promotes cell proliferation and survival, is abnormally overexpressed in numerous tumors of epithelial origin, including colorectal cancer, where it is connected with high risk of metastasis occurrence [2]. EGFR signaling cascade begins with ligand binding, what leads to the activation of tyrosine kinases. It induces activation of two main intracellular pathways – the MAPK pathway (with G protein – Ras proto-oncogene) and the PI3K/AKT pathway. These pathways lead to the activation of various transcription factors that impact on cellular responses such as proliferation, migration, differentiation, and apoptosis [3].

In this study human colon carcinoma cells BE, moving in mesenchymal way were used. Mesenchymal type of movement is Rac and Cdc42-dependent, associated with F-actin rich protrusions, extracellular matrix proteolysis, elongated morphology and often occurrence of invadopodia. Invadopodia are actin-rich structures, which are able to degrade extracellular matrix (ECM) by accumulation of matrix metalloproteinasises (MMPs) [1]. In tumours they let cells penetrate the basement membrane of blood vessels.

We have noticed that EGFR stimulation influences BE cancer cells migration. Therefore, the aim of this study was to examine the role of EGFR receptor signaling in invasion of BE cells, especially in invadopodia formation and functioning.

At first we focused on changes, which appeared in BE cells cytoskeleton architecture after EGF stimulation. Actin cytoskeleton organization and subcellular distribution of actin binding proteins (ABPs) were observed with the help of immunofluorescent staining and confocal microscopy. After EGF stimulation increased protrusions activity in relation to control cells was observed. Examined cells formed flat lamellipodia as well as invadopodia, which size and number significantly increased after stimulation. Additionally selected proteins found within invado-podia (e.g. cofilin, gelsolin, Arp3) changed their localization under these conditions. Wound healing and invasion assays on EGF stimulated cells showed that more BE cells invaded into Matrigel and they moved with higher speed. We confirmed our thesis about engagement of EGFR signaling in colon cancer cells migration by performing cytotoxic and migration tests as well as microscopic analysis on these cells after addition of EGFR inhibitors.

These results indicate that EGFR receptor signaling might be an important control mechanism which affects invadopodia functioning, and thus the migration of colon cancer cells. Moreover, because invadopodia are not critical for cell viability, therapy directed against them would be expected to have fewer side effects than classical chemo- and radiotherapy approaches.

References

SW04.S18–16
Epidermal growth factor receptor transactivation by intracellular prostaglandin E2-activated prostaglandin E2 receptors. Role in retinoic acid receptor-beta up-regulation
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In human renal proximal tubular HK-2 cells, intracellular prostaglandin E2 (iPGE2)-activated EP receptors (EPR) are responsible for the up-regulation of transcription factor retinoic acid receptor-b (RARb) upon treatment with its physiological ligand all-trans retinoic acid (ATRA). Increased expression of RARb led to transcriptional up-regulation of transcription factor hypoxia-inducible factor-1a (HIF-1a) and enhanced production of HIF-1a-regulated renoprotective factor vascular endothelial growth factor-A (VEGF-A). Here we have studied the role of transcriptional mechanisms in this experimental setting and, particularly, the mechanism involved in RARb up-regulation. We found that epidermal growth factor receptor (EGFR) transactivation by iPGE2-activated EPR plays a crucial role in RARb up-regulation since: i) PGF2 or ATRA, which are iPGE2 inducers, determined an increase in RARb mRNA expression and activity of a retinoic acid receptor response element (RARE) construct from the RARb gene promoter ii) These effects were prevented by EPR antagonists or by blocking the increase in iPGE2 with inhibitor of prostaglandin uptake transporter bromocresol green or cyclooxygenase inhibitor diclofenac iii) PGF2 and ATRA increased EGFR phosphorylation in an EPR antagonist-sensitive manner, and iv) EGFR inhibitor AG1478 prevented PGF2- and ATRA-induced increase in RARE activity and RAR expression. EGFR transactivation by iPGE2 resulted in RARb-dependent HIF-1a transcriptional up-regulation and VEGF production (presumably through activation of the hypoxia-responsive element in the VEGF-A gene promoter). Sequential activation of EPR, EGFR and RARb by iPGE2 might be the basis of new pharmacological approaches for the therapeutic modulation of HIF-1a.

SW04.S18–17
Epidermal growth factor receptor-dependent activation of MSK-1 by intracellular prostaglandin E2 results in increased production of vascular endothelial growth factor-A through up-regulation of retinoic acid receptor-b
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The pharmacological modulation of renoprotective factor vascular endothelial growth factor-A (VEGF-A) in the proximal tubule...
has therapeutic interest. In human proximal tubular HK-2 cells, treatment with all-trans retinoic acid (ATRA) or prostaglandin E₂ (PGE₂) triggers the production of VEGF-A. The pathway involves an initial increase in intracellular PGE₂ followed by activation of EP receptors (PGE₂ receptors, most likely an intracellular subset), which leads to epidermal growth factor receptor (EGFR) transactivation. This results in the sequential activation of retinoic acid receptor b (RARb) and hypoxia-inducible factor-1a (HIF-1a) leading to increased production of VEGF-A. Here we studied the role of kinases in EGFR-dependent transcriptional increase in RARb expression leading to increase in HIF-1a/VEGF-A. We found that treatment with PD98059 (which inhibits the activation of ERK) or p38 SB203580 (a p38 inhibitor) did not prevent PGE₂- or ATRA-induced EGFR phosphorylation but blunted PGE₂- or ATRA-induced increase in RARb/ HIF-1a/VEGF-A. Furthermore, the inhibitor of EGFR activation AG1478 prevented PGE₂- or ATRA-induced increase in ERK and p38 phosphorylation. We confirmed that all the inhibitors tested prevented the increase in RARb mRNA and in the activity of a retinoic acid-response element from the RARb gene promoter. These results suggested that ERK and p38 are required for EGFR-dependent transcriptional increase in RARb expression leading to increase in HIF-1a/VEGF-A. We next asked whether MSK-1, which is activated by ERK and p38 was involved in the increase in RARb expression: MSK-1 phosphorylation increased upon treatment with PGE₂ and ATRA, which was prevented by PD98059 and SB203580, and MSK-1 inhibitor H89 prevented the transcriptional increase in RARb expression induced by both treatments, as well as the consequent increase in HIF-1a/VEGF-A. These results indicate that EGFR-dependent activation of ERK and p38, resulting in MSK-1-dependent transcriptional increase in RARb, might be a target for the therapeutic modulation of HIF-1a/VEGF-A.

**SW04.S18–19**

**Study of a new potent C5aR non-competitive allosteric inhibitor for pain treatment**

E. Mayo, A. Aramini, M. M. Teixeira, G. Bianchin

One of the newest drugs used in treatment of melanoma is vemurafenib (PLX4032), an inhibitor of mutated form (V600E) of BRAF kinase, a kinase downstream of receptor tyrosine kinases (RTKs). We studied effects of vemurafenib on proliferation rate and invasion abilities of selected melanoma cell lines, A375, WM35, WM9, WM239 and Hs294T. We show vemurafenib indeed has a cytotoxic effect on melanoma cells and influences invasion ability of these cells. However, it was shown by others that the cells can be completely or partially rescued from cytotoxic effect of vemurafenib by exposure to some growth factors (GFs) such as hepatocyte growth factor (HGF) or epidermal growth factor (EGF) (Straussman et al., 2012; Wilson et al., 2012). That is why we decided to check influence of EGF, HGF and additionally tumor growth factor beta 1 (TGFbeta1) on cells proliferation rate, invasion ability and subcellular distribution in tested cells of invadopodia forming proteins: gelsolin, actin and cortactin. We decided to include in our studies TGFbeta 1 because it was shown this agent relieved from extracellular matrix has an impact on cancer cells (Hanahan et Weinberg, 2011). We have obtained startling results showing different responses of melanoma cell lines to these factors. For instance TGFbeta 1 strongly inhibited invasion of WM9 cells obtained from metastasis to lymph nodes, whereas in case of other cell lines invasion rose about 400-600% after TGFbeta 1 treatment. To further study interplay of RTKs in melanoma cells we have additionally treated the cells with inhibitors of RTKs, i.e. of EGFR (gefitinib), Met (crizotinib), and TGFbeta 1 receptor (LY 364947) and looked at the same factors as in the case of GFs treatment. We have observed that e.g. though EGFR inhibitors did not affected proliferation gefitinib affected surprisingly organization of invadopodia.

Altogether it shows tested GFs have different effects on selected melanoma cell lines suggesting origin of a cell line could play a role in response to GFs.

This work was supported by the Foundation for Polish Science (HOMING Plus/2010-2/8) within European Union Innovative Economy Programme.

**References**


**SW04.S18–19**

**Different effects of selected growth factors and RTK inhibitors on invasiveness of melanoma cells**

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One of the newest drugs used in treatment of melanoma is vemurafenib (PLX4032), an inhibitor of mutated form (V600E) of BRAF kinase, a kinase downstream of receptor tyrosine kinases (RTKs). We studied effects of vemurafenib on proliferation rate and invasion abilities of selected melanoma cell lines, A375, WM35, WM9, WM239 and Hs294T. We show vemurafenib indeed has a cytotoxic effect on melanoma cells and influences invasion ability of these cells. However, it was shown by others that the cells can be completely or partially rescued from cytotoxic effect of vemurafenib by exposure to some growth factors (GFs) such as hepatocyte growth factor (HGF) or epidermal growth factor (EGF) (Straussman *et al.*, 2012; Wilson *et al.*, 2012). That is why we decided to check influence of EGF, HGF and additionally tumor growth factor beta 1 (TGFbeta1) on cells proliferation rate, invasion ability and subcellular distribution in tested cells of invadopodia forming proteins: gelsolin, actin and cortactin. We decided to include in our studies TGFbeta 1 because it was shown this agent relieved from extracellular matrix has an impact on cancer cells (Hanahan et Weinberg, 2011). We have obtained startling results showing different responses of melanoma cell lines to these factors. For instance TGFbeta 1 strongly inhibited invasion of WM9 cells obtained from metastasis to lymph nodes, whereas in case of other cell lines invasion rose about 400-600% after TGFbeta 1 treatment. To further study interplay of RTKs in melanoma cells we have additionally treated the cells with inhibitors of RTKs, i.e. of EGFR (gefitinib), Met (crizotinib), and TGFbeta 1 receptor (LY 364947) and looked at the same factors as in the case of GFs treatment. We have observed that e.g. though EGFR inhibitors did not affected proliferation gefitinib affected surprisingly organization of invadopodia.

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**References**

SW04 Molecular Mechanisms of Disease

SW04.S18–20
Identifying key residues important for CGRP binding to its receptor
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The calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide. Its receptor is a heterodimeric complex of calcitonin receptor-like receptor (CLR) – a family B G-protein coupled receptor – and a single-pass transmembrane protein, receptor activity modifying protein 1 (RAMP1). Here, we identify residues, within the N-terminal extracellular domain (ECD) of CLR, potentially involved in ligand binding.

Certain residues presumed to be possible sites of contact for the CGRP were picked from the CLR/RAMP1 ECD crystal structure (PDB 3N7S). Residues were mutated to alanine (A) by site-directed mutagenesis (QuikChange®, Stratagene). Mutants were analysed for their ability to stimulate cAMP and cell surface expression as previously described [1].

All mutants showed reduced potency, though to varying degrees as indicated by their pEC50 values. W69A and D70A showed significant reduction in cell surface expression. These findings suggest that these residues are important for the interaction of CGRP with its receptor.

References

SW04.S18–21
Gi-protein dependent pathway and protein kinases activation contributes to alpha-fetoprotein induced THP-1 cell invasion and chemotaxis
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Human recombinant alpha-fetoprotein (rhAFP), expressed in Saccharomyces cerevisiae, was purified from the culture medium and used to study signaling mechanisms in human monoblastoid leukemia cell line THP-1. We have found that rhAFP possesses some chemokine-like activities, operating by enhancing THP-1 cell invasion in the matrix metalloproteinase (MMP)-dependent manner, triggering chemotaxis, calcium mobilization, and inducing MMP9 expression, which have been detected by zymography, Western blot and RT PCR techniques. The inhibitor of GαiPCRs pertussis toxin dramatically augments rhAFP-induced MMP9 expression and calcium response indicating that Gi protein coupled receptor(s) has a mediatory role in these processes. The action of CCR5 inhibitor Maraviroc results in partial suppression of MMP9 up-regulation and calcium response suggesting that CCR5 might be involved in these effects. By use small molecule kinase inhibitors we have shown that the MMP9 expression exerted by rhAFP in THP-1 cells depends on the activation of ERK1,2, JNK and Akt kinases. In the contrast, inhibition of p38 kinase, but not that of JNK, had dramatic suppressive effect on the rhAFP-triggered chemotaxis. Our data suggest that G-protein coupled receptor mediated mechanisms, as well as protein kinase dependent pathways are involved in AFP-triggered migration and MMP9 expression.

SW04.S18–22
Tangled in the signal network: How calcium and adhesion modulates Rho-dependent signaling?
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Cell migration is one of the crucial attributes of a living organism. It is responsible for embryogenesis, regeneration, immune defense, as well as such undesirable phenomena, as the spreading of cancer cells. Two major conditions must be fulfilled for cell migration to occur: polarization and adhesion. These processes are spatially and temporally regulated by signaling pathways related to RhoA and Rac1 proteins – the key regulators of actin cytoskeleton dynamics: contractility and polymerization, respectively.

We indicated the mutual compensation of these two signaling pathways in glioma C6 cells by blocking each of them. Under both experimental conditions stimulation of P2Y2 receptors with UTP resulted with cells recovery to control morphology and motility. We examined the differences between cell migration parameters (average velocity, walk persistence, directionality) and adhesion areas in control cells, those with blocked RhoA/ROCK or Rac/PAK pathway (by Y-27632 and NCS inhibitor respectively) and in cells in calcium-free medium. We showed that NCS and calcium-free environment prevent cell recovery from ROCK inhibition after UTP stimulation [1]. Under these experimental conditions interaction of α,β integrins with P2Y2 receptors is decreased, as microscopy and biochemical studies showed, inhibiting cofilin phosphorylation via Rac1/PAK signaling pathway [2] and changing the migration parameters. The dependence between examined signaling pathways and the pattern of glioma C6 cells migration is discussed.

References

SW04.S18–23
Thrombin-induced CCAAT/enhancer-binding protein beta activation and IL-8/CXCL8 expression is mediated by MEKK1, ERK, and p90 Ribosomal S6 Kinase 1 pathways in lung epithelial cells
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Thrombin is a well-known coagulation factor generated during vascular injury and which plays a crucial role in lung inflamma-
protein to the long list of nuclear receptors already shown to be present in diverse membrane structures.

**SW04.S18–25**

**Biochemical mechanisms involved in the modulation of dopamine signaling by Trace Amine Associated Receptor 1 (TAAR1)**

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Recent observations have indicated that G protein-coupled receptor Trace Amine Associated Receptor 1 (TAAR1) represent a novel target for the pharmacology of dopamine-related disorders such as schizophrenia, ADHD and Parkinson’s disease. To investigate the mechanisms of TAAR1-dependent modulation of physiological functions mediated by dopamine we performed series of experiments by using both in vivo and in vitro approaches. By applying various experimental paradigms aimed to model dopaminergic dysregulation in mice lacking TAAR1 and newly developed selective TAAR1 ligands, we investigated the potential role of TAAR1 in modulating dopamine-related functions such as movement control. Furthermore, we investigated the biochemical mechanism of interaction between TAAR1 and D2 dopamine receptors and the role this interaction plays in D2R-related signaling and behaviors. Finally, we applied in vivo microdialysis and fast scan cyclic voltammetry (FSCV) to investigate biochemical mechanisms involved. In TAAR1 knockout (KO) mice, we observed that TAAR1 generally exerts an inhibitory influence on the locomotion, so TAAR1 agonists inhibit dopamine-dependent locomotor activity, while effects of dopaminergic stimulation is enhanced in TAAR1 KO mice. In biochemical studies, we observed close functional interaction between TAAR1 and D2 dopamine receptors. A significant modulation of dopamine dynamics following TAAR1 activation was also observed in neurochemical studies. These data indicate that TAAR1 can affect dopamine neurotransmission via several mechanisms and this modulatory influence can have important functional consequences in vivo.

**SW04.S18–26**

**Certain cancer and RASopathy associated mutations affect Ras nanoclustering**

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Solution structures and biochemical data of the GTPase Ras have provided a wealth of mechanistic insight into the functioning of this notorious and apparently undruggable oncoprotein. However, relatively little is known about how exactly Ras functions on the plasma membrane. Hancock and co-workers elaborated that Ras is organized into signaling platforms in the membrane, termed nanocluster. Nanoclustering is required for robust Ras signalling and impacts critically on its signalling output. We previously showed that guided by a novel switch III, helix alpha 4 and the C-terminal hyper variable region stabilize guanine nucleotide-dependent orientations of membrane bound H-ras [1]. Specific mutations in these structural elements (orientation-

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switch III mutants) systematically affect effector recruitment and ensuing downstream MAPK-signaling [2].

Here we show by STED-FCS, FLIM-FRET and FRAP-experiments that orientation-switch III mutants exhibit specific anomalous diffusion and immobilization responses to cellular doses of the nanoclustering scaffold galectin-1. The mutant-specific nanoclustering-response correlates with cellular effector recruitment. Importantly, we provide first evidence that a cancer associated mutation affects Ras nanoclustering. These data emphasize the need to comprehensively understand Ras nanoclustering.

We have therefore developed a high-throughput amenable screen for novel nanocluster modulators. We validated the potential of this assay by chemical library screening and showing that it was suitable to identify the tumor suppressor and RASopathy associated SPRED1 as a novel K-ras4B-associated nanodomain modulator. Our results suggest an unprecedented mechanism of action for the negative regulatory role of SPRED1 in the Ras/MAPK-pathway.

In summary, we demonstrate a broad relevance of Ras nanoclustering in the disease context and therefore propose it as a novel drug-target.

References

SW04.S18-27
Role of non-degradative ubiquitination of the dopamine D4 receptor
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Ubiquitination is a post-translational modification that targets proteins for degradation but can also regulate other cellular processes such as endocytosis, trafficking, DNA repair, et cetera.

We are investigating ubiquitination of the dopamine D4 receptor (D4R) which belongs to the superfamily of G protein-coupled receptors (GPCRs), the most common drug targets. Several polymorphic variants of the dopamine D4 receptor exist, which differ in the number of 16-amino acid repeats in the third intracellular loop of the receptor. The functional role of the receptor polymorphic region is not known but persons with the seven-repeat allele show a predisposition to develop Attention Deficit Hyperactivity Disorder (ADHD).

We have identified a protein, KLHL12, which specifically interacts with this region and enhances ubiquitination of the D4R [1]. We have found that KLHL12 does not promote ubiquitination of D4R with seven repeats, in contrast to strong induction of ubiquitination of other tested variants. This suggests that differential ubiquitination of the D4R has functional implications. Moreover, we were able to demonstrate that KLHL12-mediated D4R ubiquitination does not lead to receptor degradation [2].

In this study we are testing the hypothesis that KLHL12 promotes ubiquitination on non-lysine residues of the D4 receptor. First, the ubiquitination pattern of the receptor with all intracellular lysines mutated to arginines was characterized. Next, we examined the importance of the cysteine and serine/threonine residues in ubiquitination process of the receptor. The obtained results seem to confirm our hypothesis but further investigation is still necessary.

Additionally, we are studying the influence of ubiquitination on receptor signaling and found a role in modulation of cAMP levels upon D4R activation. To obtain a broader picture of the involvement of ubiquitination in receptor signaling we will now perform gene expression profiling to investigate the influence of receptor ubiquitination on specific cAMP and calcium inducible genes.

References

SW04.S18-28
Lysophosphatic acid induces rapid myosin-9/tropomyosin complex rearrangement and myosin-9 cleavage in human fetal lung fibroblasts
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Lysophosphatic acid (LPA) is a bioactive lipid mediator that acts via specific G-protein-coupled receptors. LPA activates Rho/ROCK pathway resulting in actin cytoskeletal rearrangements. GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho. Myosin-9 (Nonmuscle myosin IIa) is a motor protein with RhoGAP-activity. It is associated with the wide range of cellular functions, including cell shape and motility. We have previously demonstrated that myosin-9 forms multi-molecular complexes with high molecular weight tropomyosin (TM) isoforms and heat shock proteins in cytoplasm of cultured cells [1]. Considering that LPA is known to activate Rho, and myosin-9 is known to inhibit Rho, we explored the myosin-9 and TM levels at different timepoints after LPA (20 ng/ml) stimulation. Cytosol extracts from human embryonic lung fibroblasts were subjected to co-immunoprecipitation assay with polyclonal antibodies specific to C-terminal peptide of human myosin-9, or with monoclonal antibodies recognizing TM1, 2, 3, and 6. We have observed the significant decrease in myosin-9 co-immunoprecipitated TM level immediately (30 s) after LPA addition. Gel-filtration chromatography confirmed the myosin-9/tropomyosin complex disassembly after LPA treatment. Additionally, we have found that LPA stimulation induces myosin-9 degradation with accumulation of 130 kDa fragment in cytomatrix fraction. Finally, double immunofluorescence and confocal microscopy analysis demonstrated a decrease in colocalization of TM with myosin-9 in cells during 10 min following LPA induction. We suggest that TM inhibits myosin-9 RhoGAP-activity by interacting with its RhoGAP domain. Further studies are needed to elucidate the molecular mechanisms responsible for this interaction.

Reference
ROCK inhibitor Y-27632 increases invasion of HeLa cell by Serratia spp.
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Serratia are common nonpathogenic bacteria that can cause opportunistic infections (Hertle et al., 1994). We have previously found that S. grimesii and S. proteamaculans are capable to invade eukaryotic cells provided that they synthesize intracellular metalloprotease grimeysin or protealysin, respectively. Moreover, expression of grimeysin or protealysin gene in non-invasive E. coli confers an invasive phenotype (Bozhokina et al., 2011). However, the mechanisms used by these bacteria to invade non-professional phagocytic cells are so far poorly characterized. One of the factors involved in the invasion was shown to be E-cadherin whose higher expression stimulated by N-acetylcysteine correlated with the increasing entry of grimeysin producing bacteria into HeLa cells (Bozhokina et al., 2013). N-acetylcysteine is also known to regulate both Rho and Src kinase pathways. Therefore in this work we used Y-27632, a selective inhibitor of the Rho-associated protein kinase p160ROCK, and Src kinase inhibitor-1 to reveal their effect on invasion of the bacteria producing grimeysin and protealysin. Incubation of HeLa cells with 5 or 10 mM Y-27632 caused changes in morphology of HeLa cells, including actin cytoskeleton disassembly. These changes are accompanied by a twofold increase of invasion by Serratia grimesii and S. proteamaculans, and a threefold increase of invasion by recombinant E. coli SCS1. These effects are similar to the effects observed in the N-acetylcysteine-treated HeLa cells. In contrast, inhibition of Src kinase led to a significant (up to eightfold) decrease of S. grimesii invasion. The inhibition was also observed when 10 mM Src inhibitor-1 was added together with 10 mM Y-27632, thus demonstrating an essential role of this enzyme in Serratia invasion. Taken together, these results indicate that both ROCK and Src kinase control susceptibility of HeLa cells to Serratia invasion, and the downstream pathways regulated by these kinases are different. This work was supported by RFBR 12-04-31109 and OPTEK young scientist grants.

Involvement of EGFR signaling in colon cancer cell migration
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Epidermal growth factor (EGF) induces formation of cell protrusions associated with actin cytoskeleton remodeling and cell migration [1]. Epidermal growth factor receptor (EGFR) promotes cell proliferation and survival. It is overexpressed in colorectal cancer, where it is connected with high risk of metastasis occurrence [2]. EGFR signaling cascade induces activation of two main intracellular pathways – MAPK pathway and PI3K/AKT pathway. These pathways lead to the activation of various transcription factors that impact on cell proliferation and migration [3].

In this study human colon carcinoma cells BE, moving in mesenchymal way, were used. This type of movement is associated with extracellular matrix proteolysis and occurrence of invadopodia. Invadopodia are actin-rich structures, which can degrade extracellular matrix by accumulation of matrix metalloproteinases [1]. They let tumour cells penetrate the basement membrane of blood vessels.

The aim of this study was to examine the role of EGFR signaling in invasion of BE cells, especially in invadopodia formation and functioning.

At first we focused on changes, which appeared in BE cells cytoskeleton architecture after EGF stimulation. Examined cells formed flat lamellipodia as well as invadopodia, which size and number significantly increased after stimulation. Additionally selected proteins found within invadopodia changed their localization under these conditions. Wound healing and invasion assays on EGF stimulated cells showed that more BE cells invaded into Matrigel and they moved with higher speed. We confirmed our thesis about engagement of EGFR signaling in colon cancer cells migration by performing cytotoxic and migration tests as well as microscopic analysis on these cells after addition of EGFR inhibitors.

These results indicate that EGFR receptor signaling might be an important control mechanism which affects invadopodia functioning, and thus migration of colon cancer cells. Moreover, because invadopodia are not critical for cell viability, therapy directed against them could trigger fewer side effects than classical anti-cancer therapy.

References

G protein-coupled receptors in atherosclerosis: cannabinoid receptors as novel modulators of atherogenesis
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Atherosclerosis is a progressive and chronic inflammatory disease which is the primary cause of morbidity and mortality in the western world and whose identification of promising novel therapeutic strategies is thus of great interest. The endocannabinoid system (ECS) comprises a class of endogenous bioactive lipids, the cannabinoid receptors 1 and 2 (CB1R and CB2R), that belong to the G protein-coupled receptors (GPCRs) family, and the enzymatic machinery deputed for endocannabinoids metabolism. The role of cannabinoid receptors in atherosclerosis is still controversial: while CB1R is closely associated with cardiometabolic risk factors, including obesity and increased lipid production/accumulation, CB2R seems to bear a protective and anti-inflammatory role, downregulating both innate and adaptive immune responses. However, some of the well-documented non-CB1R, non-CB2R effects of certain cannabinoid ligands have recently attributed to the orphan G protein-coupled receptor GPR55, alias the ‘purposed CB3’ receptor. GPR55 was firstly associated with several physiological processes, including vascular relaxation, glucose homeostasis and immune responses. Our work investigates for the first time the expression and the effects exerted by cannabinoid receptors in human atherosclerotic cells, inasmuch as we observed differential effects in regulating the receptor-mediated influx/efflux of cholesterol from foam cells. Furthermore, we show an unprecedented effect of these receptors in modulating several inflammatory immune responses, with each receptors showing a distinct cytokine profile. Overall, we provide evidence that the ECS is indeed a novel biomarker of atherosclerosis and that it is implicated in modulating several key hallmarks of this disease; thus our findings may be of crucial importance for the rational design of new endocannabinoid-based immunotherapeutic strategies for atherosclerosis or cardiovascular diseases.
The spatial and temporal organization of signaling networks in response to distinct stimuli, may influence substrate specificity and biological outcomes. Deregelation of these signaling complexes is associated with a large range of pathological disorders. In particular ERK1/2 signaling abnormalities, resulting most often from Ras or Raf mutations, occur in about 30% of human cancers. Our work shows that the ERK1/2 pathway takes centre stage in pituitary function. The small GTPases Ras and Rap1, those operate as molecular switches upstream of the Raf/MEK/ERK cascade, are differentially recruited and activated by growth factors (as EGF) and Neuropeptides [as vasoactive intestinal polypeptide (VIP)]. Using single cell imaging and the genetically encoded Fluorescence Resonance Energy Transfer (FRET)-based sensor of ERK activity (EKAR) we follow the spatiotemporal dynamic of ERK activation. We establish for the first time that two physiological modulators of pituitary function (EGF and VIP) finely regulate the dynamic of cytoplasmic and nuclear pools of activated ERK in living cells. Ras and Rap1, that encode the properties of the activated receptors, play a key role in shaping ERK response kinetics in both compartments.

The differential recruitment of those monomeric G proteins, which specifically controls the cytoplasmic and nuclear pools of activated ERK, is responsible for the subtle regulation of the prolactin (PRL) gene. EGF-dependent Ras activation, which exclusively determines the nuclear magnitude and duration of ERK activation, increases PRL transcription. VIP-dependent Ras activation which controls both nuclear and cytoplasmic magnitude and duration of ERK activation acts as a repressor of the PRL gene regulation. On the contrary, VIP-dependent Rap1 activation, which only dictates the nuclear magnitude and duration of ERK activation, leads to an increase in PRL transcription.

Finely, we establish that inductive expression of the gsp oncogene (constitutive activation of the heterotrimeric Gs protein observed in somatotroph adenomas) initiates a PKA-dependent sustained ERK activation and hormonal hypersecretion. In this pathological context, Ras is switched to an activator of the PRL gene (constitutive activation of the heterotrimeric Gs protein) which specifically controls the cytoplasmic and nuclear pools of activated ERK.

The role of endocannabinoids in early events of endothelium/leukocyte interactions.

2-Arachidonoylglycerol (2-AG) induces the expression of selectins and their ligands involved in leukocyte capture and rolling, thus modulating the leukocyte adhesion cascade. Brief exposure of endothelial cells to 2-AG prime them towards a pro-inflammatory state, characterized by time-dependent plasma membrane expression of P- and E-selectins and tumour necrosis factor-α (TNF-α) release. Commitment to inflammation is permanent, since TNF-α secretion is observed for up to 24 hours, despite the removal of 2-AG after 1 hour of incubation. TNF-α-containing medium, then, promotes leukocyte recruitment, characterized by enhanced L-selectin and P-selectin glycoprotein ligand-1 (PSGL1, the specific ligand for selectins) expression, as well as increased efficiency of adhesion and trans-migration.

In conclusion, 2-AG regulates cell-to-cell cross-talk, representing a potential therapeutic target to treat inflammatory diseases.
The interaction between the dopamine D₂ and the serotonin 5-HT₂A receptors plays an essential role in schizophrenia (affects about 1% of human population). Both of these receptors have been implicated as important site of action of antipsychotics such as clozapine due to well documented serotonin-dopamine interaction and its relevance to schizophrenia. In the light of recent data (Lukasiewicz 2011) selective action of clozapine only on heteromers D₂-5-HT₂A may have better therapeutic properties then when the drug interacts via mono- or homomers which may have a key significance in novel therapy of schizophrenia. Unfortunately, currently available antipsychotics – such as clozapine – addressed only for tiny part of cell population in the brain are non-specifically delivered into all brain areas, causing undesirable side effects. Moreover, clozapine have poor oral bioavailability (<27%). Keeping this in mind, the evaluation of the way of selective regulation of D₂-5-HT₂A heterocomplexes seems to be very important. Currently nanoparticulate system with functionalized surfaces for targeted delivery plays a central role in modern therapies. The application of nanoparticulate pharmaceutical carriers increases bioavailability and efficiency of many drugs. Surface modification of nanocarriers is used to control their biological properties in a desirable fashion and to enable them to perform therapeutically or diagnostically important functions in proper place and at right time. The nano-strategy also leads to the lowering of drug dose – reducing unfavorable side effects. Therefore, the aim of the study is development of new form of clozapine specific for D₂-5-HT₂A heteromer. Clozapine nanocapsules will be functionalized by using synthesized human scFv antibody. The antibody is able to specifically recognize the heteromer formed by both receptors, and simultaneously it is not able to bind to the monomeric form of receptors. To obtain such properties, the antibody should recognize structural, spatial epitope formed within heteromer structure.

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Reference

Objective: Previous many studies suggest that early nutrition during critical developmental periods affects long-term health. Infants of diabetic mothers (IDM) have also abnormal circulatory organs. When we investigated the insulin signaling in the newborn rat heart, they found that the insulin signaling showed insulin resistance at the Akt/mTOR pathway. However, we have already reported that the abnormality of insulin signaling of the infant hearts of diabetic mothers were improved by feeding the pregnant mothers a diet rich in fish oil. In this study, we would like to clarify that what kind of ingredient of the fish-oil improves insulin signaling by the primary neonatal cardiomyocyte (PNCC).

Methods: Pregnant diabetic rats induced by streptozotocin and were then fed via gastric tube EPA (EPA group) or a control DDW (control). To examine changes in insulin signaling in the cardiac muscle in IDM, we isolated the heart and cultured monolayer PNCC. Western blotting was carried out for determine the phosphorylation levels of Akt and the expression levels of mTOR and GLUT-4. We also confirmed the Akt/mTOR pathway using the H2C9 rat cardiomyoblast cell line.

Results: In cardiac muscle and PNCC, the phosphorylation level of Akt and the expression level of mTOR and GLUT-4 were decreased in IDM of the control group. However, the phosphorylation level of Akt and the expression level of mTOR were increased in the EPA group compared with control group. Furthermore, the results were confirmed by H9C2 cell line.

Conclusion: The EPA in the fish oil may improve the impaired signaling pathway of the cardiac muscle of infants caused by a diabetic mother’s hyperglycemia.

The role of mTOR signal pathway in rat ovulation and implantation

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Mtor is a master kinase that regulates several critical intracellular processes and includes PI3K/AKT/mTOR signal pathway. It affects several cell functions, including growth, proliferation, protein synthesis and transcription, angiogenesis, apoptosis and autophagy. A lot of molecules as ERK, RHO/ROCK, PTK2 and PI3K/AKT/mTOR play an important role in achievement of ovulation and implantation. In this study, our aim was to evaluate mTOR and associated molecules in rat ovary and uterus at ovulation and implantation processes by using indirect immunohistochemical and Western blotting methods.

Female rats (n:49) were divided into seven groups according to their vaginal smear examinations as proestrous, estrous, metestrous, diestrous groups and 4.5, 5.5 and 6.5 embryonic days (ED) of pregnancies. The samples were fixed in 10% formaline solution and prepared according to the routine paraffin tissue protocol and then were embedded in paraffin. Sections were
immunostained with anti-mTORC1, anti-mTORC2, anti-IGF1, anti-P13K, anti-pAKT1/2, anti-ERK1 and anti-pERK1/2 primary antibodies by using the avidin-biotin-peroxidase immunohistochemistry method. Anti-pAKT1/2/3 and anti-ERK1 primary antibodies were confirmed by using the Western blotting technique. Immunoreactivity intensity scores were determined as mild, moderate or strong by using the semi-quantitative method. The results were compared with the ANOVA statistical test.

In the evaluation of all the groups, it was observed that all primary antibodies increased in the proestrus and eustrous groups in ovulation and 6.5 ED in implantation processes. The highest immunoreactivity was seen in anti-ERK1 primary antibody and then gradually decreased in anti-mTORC1, anti-pAKT1/2/3, anti-IGF1, anti-pERK1/2, anti-P13K and anti-mTORC2 primary antibodies. The existences of anti-pAKT1/2/3 and anti-ERK1 primary antibodies were confirmed by using the Western blotting technique.

In this study, mTOR and associated molecules were evaluated in the processes of rat ovulation and implantation and it was thought that they might play an important key role in these processes. It is considered that P13K/AKT/mTOR and IGF molecules might be used in assisted reproductive techniques, in the treatment of infertility and thus might increase the chances in clinical success.

**SW04.S18–39**

Direct observation of G-protein coupled receptor heteroreceptor complexes in the brain by in situ proximity ligation assay


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G protein–coupled receptors (GPCRs) play critical roles in cellular processes and signaling and have been shown to form heteromers with diverge biochemical and/or pharmacological activities that are different from those of the corresponding monomers or homomers [1,2]. The idea of the existence of direct interactions between two different GPCRs at the level of the plasma membrane has its origin in 1980/81 on the basis of the discovery that peptides like cholecystokinin-8 [CCK-8] and substance P could modulate the density, and especially the affinity, of distinct monoamine receptors in membrane preparations from the Central Nervous System with in vivo functional correlates [3,4]. These initial findings were in line with the previous discovery of negative cooperativity between β-adrenergic receptors in 1974/75 by Lefkowitz, Limbird and colleagues indicating the possible existence of homodimers of β adrenergic receptors leading to site–site interactions in recognition [5]. However, despite extensive experimental results supporting the formation of GPCR heteromers in heterologous systems, the existence of such heteroreceptor complexes in their native environment remains largely unknown, mostly because of the lack of appropriate methodology. In order to demonstrate in native tissue the existence of GPCR heteromers, especially in a manner that can be generally applicable to different receptor pairs, a well-characterized in situ proximity ligation assay [in situ PLA] has been adapted to confirm the existence of GPCR heteroreceptor complexes in brain slices ex vivo. In addition, we describe the in situ PLA procedure as a high selectivity and sensitivity assay to image GPCR heteromers in brain sections by confocal microscopy and discuss advantages and disadvantages of this method compared to other available techniques.

**References**


Feedback loops controlling the GDP/GTP cycle of Arf1 on membranes
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The small G protein from Arf1 controls numerous effector proteins at the Golgi complex. This includes the protein coat, COPI, which is involved in the formation of transport vesicles, the long string-like protein GMAP-210, which tethers cisternae and vesicles, and the sterol transporter OSBP. These effectors are functionally and structurally very different. We study their spatiotemporal regulation using reconstitution experiments with purified proteins and artificial membranes. These experiments reveal feedback loops, whereby the changes in membrane chemistry and curvature that result from the formation of Arf-effector complexes, control, in turn, the GDP/GTP switch of Arf. These regulatory mechanisms explain how the Golgi keeps a constant lipid composition and well-organized membrane architecture despite the intense membrane traffic that passes through it.

Investigating the molecular mechanisms underlying the differential subcellular targeting of the metabotropic glutamate receptor 1 (mGlu1) in the cerebellar cortex
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Type 1 metabotropic glutamate (mGlu1) receptor activates a multitude of signalling pathways important for modulation of excitability and plasticity in the cerebellar cortex. mGlu1 receptor is involved in long-term depression at excitatory inputs to Purkinje cells (PC), whereas it is implicated in rebound potentiation of inhibitory post-synaptic potentials at GABAergic synapses. These various forms of plasticity might depend on subsynaptic arrangement of the receptor that can be regulated by protein–protein interactions. We have taken advantage from SDS-digested Freeze-fracture replica labelling (SDS-FRL) technique and studied the distribution pattern of mGlu1 receptor in the rat and mouse cerebellar cortex. The results we have obtained following both N-terminus and C-terminus immunogold labelling confirm the perisynaptic enrichment of mGlu1 receptors, in particular of the longest splice variant mGlu1α, in excitatory synapses between parallel fibres and PCs. Our double immunogold labelling for mGlu1α receptor and GABAα3β1 subunit shows that besides excitatory synapses mGlu1α receptor is located also in the main body of GABAergic synapses in the cerebellar cortex. A well-described functional regulation of mGlu1α receptor occurs via its direct interaction with Homer proteins that are abundantly present at PCs’ excitatory inputs. We studied the possible role of long Homer proteins on mGlu1α receptor localization by using TAT-Homer1a as a dominant-negative to disrupt Homer-associated protein complexes. Our SDS-FRL analysis showed no significant difference in the perisynaptic distribution pattern of the receptor, suggesting that other scaffolding proteins are involved in the subcellular targeting of mGlu1 receptors. In order to identify other interaction partners that may regulate the targeting of the receptor in GABAergic synapses, we are currently using a proteomic approach, namely co-immunoprecipitation (Co-IP). Solubilization of high-speed membrane fractions of both wild-type and mGlu1 receptor knockout mice is carried out in buffer containing 1% non-ionic and 0.1% ionic detergent. Co-IP of mGlu1α receptor is performed using affinity-purified antibodies against the C-tail of the receptor and the eluted proteins are analyzed by quantitative mass spectrometry.

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Modeling of Parkinson’s disease and hyperprolactinemia with focus on the mechanisms of brain plasticity
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Progressive degeneration of dopaminergic (DA-ergic) neurons of the nigrostriatal system and tuberoinfundibular system of the brain is the key component of the pathogenesis of Parkinson’s disease (PD) and hyperprolactinemia, respectively. Both diseases are developed for many years up to the reaching a threshold degeneration of DA-ergic neurons with no manifestations of motor dysfunction or impairment of reproduction due to development of compensatory processes. The aim of this study was the experimental modeling of the above diseases for a study of the mechanisms of the brain plasticity serving to compensate the failure of degenerated DA-ergic neurons. The presymptomatic and early symptomatic stages of PD were modeled in mice by using l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, pronoextractin, converted to toxin in the brain. An increase of the tyrosine hydroxylase (TH) gene expression, TH activity, DA release and DA turnover observed in mice at the presymptomatic stage were considered as compensatory processes preventing motor dysfunction. In turn, a transition to the symptomatic stage appeared to be provoked by a decrease of DA release and an increase of DA uptake resulted in a drop of the DA intracellular concentration despite an increase of TH activity and hence DA synthesis. Note-worthy, the DA-ergic neuron degeneration was accompanied by an increase of the number of striatal nerve fibers containing only TH or only aromatic L-amino acid decarboxylase (AADC) and by the onset of TH gene expression in the striatal neurons. These monoenzymatic neurons were shown to synthesize DA in cooperation in the tuberoinfundibular system of rats. It means that L-DOPA synthesized in monoenzymatic TH neurons is released and captured by the monoenzymatic AADC neurons for DA synthesis. Initial DA deficiency and related hyperprolactinemia provoked by 6-hydroxydopamine-induced degeneration of the tuberoinfundibular DA-ergic neurons were compensated with time due to the increase of the number of monoenzymatic neurons and apparently of DA cooperative synthesis.
The amyloid precursor protein: biochemical enigma in brain development, function and disease

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For 20 years the amyloid cascade hypothesis of Alzheimer disease (AD) has placed the amyloid-β peptide (Aβ), formed from the amyloid precursor protein (APP), centre stage in the process of neurodegeneration. However, no new therapeutic agents have reached the clinic based on exploitation of the hypothesis. It is now well documented that APP is processed in two different catabolic pathways: a minor amyloidogenic pathway in which APP is cleaved by β- and γ-secretases releasing Aβ peptide and a predominant (>90%) non-amyloidogenic pathway in which the protein is successively cleaved by α- and β-secretases precluding production of Aβ. Soluble, extracellular domains of APP are also released following the actions of α-secretase (sAPPα) and β-secretase (sAPPβ). The various APP metabolites could all have distinct physiological functions, including Aβ, which may play roles as diverse as ion channel regulation, control of haemostasis and transcriptional activation. In both catabolic pathways, the γ-secretase-mediated intramembrane cleavage of APP, after α- or β-secretase action, releases the APP intracellular domain (AICD), which can regulate transcription of several genes, including APP itself, the β-secretase BACE-1 and the Aβ-clearing proteins, neprilysin (NEP) and transisthyretin (TTR). There are three major isoforms of APP (APP695, APP751, APP770) generated by alternative splicing but, in the brain, APP695 is principally neuronal and expressed at relatively high levels compared with the other two isoforms. Although all APP isoforms are potentially amyloidogenic, in neuronal cell lines, sAPPβ, Aβ and transcriptionally active AICD are preferentially formed from the neuronal APP695 isoform. Despite intensive research, APP functions remain unclear. APP knockout mice have provided new opportunities for therapeutic intervention in AD.

Molecular determinants of Alzheimer’s disease

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In Alzheimer’s disease (AD) misfolded Aβ peptides form soluble neurotoxic oligomers in bodily fluids and eventually accumulate as extracellular aggregates in the brain. Zinc ions play a key role in pathological aggregation of Aβ and therefore affect AD pathogenesis. We have shown that region 1–16 constitutes the metal-binding domain of human Aβ. We have demonstrated the critical role of a tetrapeptide stretch 11–14, which acts as the primary zinc recognition site, in mediating zinc-dependent Aβ dimerization. In contrast with the human Aβ, rat/mouse Aβ possesses three amino acid changes in the domain 1–16, and also rats/mice are the only mammals resistant to AD. We have solved the 3D structure of the rat Aβ domain 1–16 and found that it forms zinc-bound dimer. Overall topology of this dimer prevents formation of zinc-dependent rat Aβ oligomers thus protecting rats and mice from AD. Altogether, these findings allowed us to hypothesize that conformational transitions in the domain 1–16, e.g. caused by zinc binding or amino acid chemical modifications, are responsible for triggering AD. In support of this hypothesis, we have found that isomerization of Aspartate 7, the most abundant aging-associated spontaneous chemical modification of Aβ, leads to serious structure and function changes in the region 1–16. Namely, isoAsp7-containing Aβ (isoAβ) significantly differs from the intact Aβ by better hydrolysis by the angiotensin converting enzyme, changes in zinc and copper ion chelation, and higher susceptibility to zinc-dependent oligomerization. Since each of these molecular processes is closely related to the aggregation ability of Aβ, we assumed that isoAβ would be a pathogenic agent of AD. We have found that intravenous administration of synthetic peptide corresponding to isoAβ robustly accelerates formation of classic dense-core congophilic amyloid plaques in the brain of β-amyloid precursor protein transgenic mice. Taken in the framework of the amyloid hypothesis our findings implicate this peptide as a cause of Alzheimer’s disease, and potential human blood biomarker and drug target for this neurodegenerative disorder.

Mitochondrial abnormalities as a mechanistic link in diabetes and Alzheimer disease interaction

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Type 2 diabetes (T2D) and Alzheimer Disease (AD) are major health problems affecting our society as a consequence of an aged population, especially in developed countries, as a result of improvement in life style. Epidemiological studies showed that a close association exists between the two pathologies with T2D being considered, together with aging, the major risk factor for AD development. We hypothesize that mitochondrial alterations can be in the beginning of the alterations that will lead to neuronal cell death. Therefore this study was aimed to evaluate and compare the effects of AD and sucrose-induced metabolic alterations on brain mitochondrial function and how it affects behavioral and cognitive function as well as in AD Neuropathological markers. To achieve our goal we performed in vivo studies were we provided 20% sucrose-sweetened water to wild-type (WT) mice for 7 months. WT and triple transgenic Alzheimer’s disease (3xTg-AD) mice without access to sucrose-sweetened water were also used. Several parameters were evaluated such as brain mitochondrial function, fear and anxiety, learning and memory abilities, Abeta peptide levels both in cortex and hippocampus. We observed that sucrose intake increased body weight, postprandial and occasional glucose, glycated haemoglobin (HBA1c) and tri-glycerides levels and decreased brain weight. 3xTg-AD mice presented an increase in postprandial glucose and HBA1c levels and a decrease in brain and body weight. In addition, we observed a dysfunction in brain mitochondrial function in both WT sucrose-treated and 3xTg-AD mice when compared with WT control mice. Furthermore, a decrease in cognitive and learning abilities and an increase in AD pathological markers was observed. Overall our results show that a pre-diabetic state present a brain mitochondrial dysfunction and cell death with concomitant cognitive and behavioral alterations similar to those observed in AD. Thus, this study supports the idea that brain mitochondria are a func-
tional link between AD and (pre)diabetes. Furthermore, these oxidative and mitochondrial alterations are associated with an increase in the AD pathogenic proteins corroborating that (pre)diabetes increases the risk of developing AD.

SW04.S19–5
Interaction of Aβ40 and Aβ42 peptides with dipeptidyl peptidase IV
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The aggregates of amyloid beta peptides (Aβs) are considered as one of the main pathological hallmarks of Alzheimer’s disease (AD). It was shown that the synthesized Aβs with N-terminal deletions exhibited enhanced peptide aggregation relative to full-length species. An imbalance between the rates of synthesis and clearance of Aβs is now considered a possible contributor to the onset of AD. A number of peptidases have been shown to be capable of cleaving Aβs. A widely distributed Dipeptidyl peptidase IV (DPPIV) is a unique serine protease removing N-terminal dipeptides from the polypeptides and proteins, containing on the penultimate residue proline or alanine. Its known natural substrates are chemokines, neuropeptides, hormones and growth factors, the biological activity of which can be modulated by the enzyme. Alanine is N-terminal penultimate residue in Aβs and we presumed that DPPIV can cleave them. Cleaving of commercial Aβ40 and Aβ42 was registered by increasing of absorbance at 334 nm due to complex formation between primary amines with logical activity of which can be modulated by the enzyme.

Animal experiments support the concept that the number of D2 receptors is one of the determinants of a response to drugs of abuse.

The purpose of this study was to examine the effect of chronic prenatal exposure of MA (5 mg/kg during gestation) or the same volume of saline (S) in combination with chronic administration of MA (1 mg/kg) or S postnatal on Wistar rats. The effects of MA on striatal dopaminergic system (D1 and D2 receptors estimated by means of [3H]-SCH23390 and [3H]-spiperone specific binding) were evaluated. We compared totally four groups: (i) prenatal and postnatal saline application (S/S); (ii) prenatal saline and postnatal MA (S/MA); (iii) prenatal MA and postnatal saline (MA/S); (iv) prenatal and postnatal MA (MA/MA). Our results obtained by means of [3H]-SCH23390 are as follows: (i) we have found the decrease in the specific binding to 72% in female group (S/MA) but the increase to 120% in male group (S/MA) when compared to corresponding controls, (ii) Scatchard analysis revealed that the changes observed in females are associated with a drop in the number of binding sites, on the contrary, those in males with the higher affinity of isotope to its binding site. In conclusion, we have found that the chronic postnatal application of MA evoked the significant sex-dependent changes in D1 receptors in the striatum. Our experiments also suggest that the prenatal exposure of MA probably influences striatal D1 receptors in male rats and this trend is in accordance with published amphetamine data. Results have indicated possible difference in the binding of [3H]-spiperone to D2 receptor in striatum of males and females (MA/S) groups, namely, the binding was moderately increased in males but decreased in females. However, the differences were not statistically significant despite of the fact that the groups were enlarged. Therefore, the gender differences in D2 receptors were not confirmed.

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SW04.S19–7
Platelets early apoptosis relationship with eNOS in stroke
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Stroke is a disease that affects the blood vessels that supply blood to the brain. Platelets are implicated in its pathophysiology and is still not clear and antiplatelet agents available for the prevention and treatment of stroke. Platelets produce NO in smaller amounts than the endothelial cells and NOS isoforms have been described in platelets, but eNOS is the predominant form. We herein examined platelet activation and apoptosis relationship with eNOS in acute ischemic stroke patients. We identified 62 stroke patients who were admitted to our department and had not taken any antiplatelet drug for the prior 14 days and 44 participants for control groups. Annexin-V and P-selectin (CD62p) binding with flowcytometry, NOS activity with ELISA, eNOS gene expression with qRT-PCR, eNOS protein expression with western blotting were determined in platelets. The results showed significant increase of platelet Annexin-V (<p< 0.01) and CD62p binding (<p< 0.01) and decrease of NOS activity (<p< 0.001) in stroke patients compared to control. After stroke patients eNOS gene expression (<p< 0.05) and eNOS protein expression (<p< 0.001) releasing significantly decreased as compared with the control group. NO prevents adhesion of platelets to the vessel wall and inhibits platelet aggregation. The data of this work suggest that decreased nitric oxide may have a role in platelet early...
apoptosis in stroke which may initiate a new aspect of the role of antithrombotic drug in the treatment of acute ischemic stroke.

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SW04.S19–8
TDP-43 inclusion bodies formed in bacteria are structurally amorphous, non-amyloid and inherently toxic to neuroblastoma cells
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Accumulation of ubiquitin-positive, tau- and a-synuclein-negative intracellular inclusions of TAR DNA binding protein (TDP-43) in the central nervous system represents the major hallmark correlated to amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U). Such inclusions have variably been described as amorphous aggregates or more structured deposits having an amyloid structure. Therefore, it is not yet clear the structure adopted by TDP-43 in such deposits. Following the increasing observations that bacterial inclusion bodies generally consist of amyloid aggregates, we have overexpressed TDP-43 in E. coli cells, purified the resulting TDP-43 containing inclusion bodies (TDP-43 IBs) and subjected them to a number of biophysical analyses to assess their structure and morphology, along with control IBs. We show that the TDP-43 aggregates contained in the bacterial IBs do not bind thioflavin T and Congo red, possess a largely disordered secondary structure, as determined with circular dichroism and infrared spectroscopy, and are highly susceptible to proteinase K digestion, thus possessing none of the distinctive hallmarks for amyloid. In addition, atomic force microscopy imaging revealed that TDP-43 IBs have an irregular structure and a rough surface. However, the TDP-43 IBs were able to severely impair the viability of cultured neuroblastoma cells, when added to their extracellular medium and, even more markedly, when transfected into their cytosol. These data reveal an inherently high propensity of TDP-43 to form amorphous aggregates, which possess, however, an inherently high ability to cause cell dysfunction. This indicates that a gain of toxic function caused by TDP-43 deposits is effective in TDP-43 pathologies, in addition to possible loss of function mechanisms originating from the cellular mistrafficking of the protein.

SW04.S19–9
Aβ42 traffic through plasma membrane: role of P-glycoprotein
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Alzheimer’s disease (AD) is the most common neurodegenerative pathology that affects more than 25 millions of older people around the world. Although the cause of AD is unknown, the amyloid cascade hypothesis postulates that the deposition of the amyloid-β peptide (Aβ42) in the brain is a central event in Alzheimer’s disease pathology. This hypothesis proposes that the reason for the Aβ42 deposition is an imbalance between the production at neuronal level and its elimination trough the blood-brain-barrier (BBB). In this context, it has been proposed that P-glycoprotein (P-gp), expressed in endothelial cells of the BBB, plays a role in the elimination of Aβ42. However, the role of the P-gp remains controversial.

The aim of this study is to establish whether Aβ42 is transported by P-glycoprotein or not. The first and very important point is the method for Aβ42 solubilization. The nature of used solvent as well as the conditions of solubilization plays an important role in the behavior of the peptide in solution, which directly affects his way of spontaneous aggregation.

After the analysis of an important number of bibliographic sources, we established a protocol for the solubilization of synthetic amyloid peptide with a preliminary use of organic solvents hexafluoroisopropanol (HFIP) and DMSO. The kinetics of the aggregation of Aβ42 was followed by SDS PAGE and microscopy using Thioflavin T.

The toxicity of Aβ42 was studied. For this purpose, K562 cells overexpressing or not P-gp (K562/ADR and K562 respectively) were incubated during 3 days with the peptide solution aggregated during 24 hours. Our results show that the toxicity is similar between the two cells lines. Furthermore, we have study the transport of the pirarubicin by the P-gp in the presence or absence of Aβ42. The transport of this P-gp substrate was not modified, suggesting that P-glycoprotein is not involved in Aβ42 transport.

SW04.S19–10
Study of the process of transthyretin aggregation in presence and absence of polyphenols and other molecules
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Transthyretin (TTR) is a plasma protein secreted by hepatocytes into the blood and cerebrospinal fluid, where it transports thyroid hormones, thyroxine (T4) and triiodothyronine (T3) and cotransport of vitamin A with Retinol Binding Protein (RBP). The TTR is an amyloidogenic protein implicated in diseases such as senile systemic amyloidosis (SSA) and familial amyloid polyneuropathy (FAP), both characterized by extracellular deposition of insoluble amyloid fibrils in heart, peripheral nerves and other organs. In particular, fibrils in FAP patients are composed of single-site mutant TTR and among the numerous pathogenic variants Leu55 → Pro55 (L55P) is the most amyloidogenic and it forms amyloid fibrils in vitro. It is suspected that the single-point mutations accelerate amyloidogenesis by destabilizing the monomeric partially unfolded amyloidogenic intermediate state rather than by altering the tetrameric native state. TTR fibrils have been considered direct responsible of tissue impairment in FAP and SSA, but the unstable fibril precursors are increasingly considered the main responsible of cell suffering and tissue impairment in amyloid diseases. In particular, the early unstable oligomeric intermediates are highly toxic due to their ability to interact, disassemble and permeabilize cell membranes. Moreover, increasing information on polymorphism of pre-fibrillar and fibrillar assemblies has led to propose that apparently similar fibrils can display different stability and efficiency in generating toxic species. These data suggest the opportunity to search natural or synthetic molecules interfering with amyloid aggregation by stabilizing the TTR native state by hindering the appearance of toxic species, or by favoring the growth of less toxic assemblies. We have recently described a natural compound (oleuropein) which is protective in Tg animal models of Abeta deposition and cultured cells by stim-
ulating cell autophagy and the endolysosomal path and by modifying the pattern of aggregation of amylin and Abeta peptides skipping the appearance of toxic oligomers and reducing plaque load. Our study is focused on the ability of oleuropein (the main phenolic component of Mediterranean extra virgin olive oil) to inhibit the aggregation process in both wild-type amyloid TTR and highly amyloidogenic L55P variant. Our data offer the possibility to validate and optimize the use of rationally designed and promising drugs that could enter in a clinical experimental phase.

**SW04.S19–11**

**Extramitochondrial oxidative phosphorylation in myelin sheath: Reactive oxygen species production and axonal degeneration in demyelinating diseases**

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Myelin has been historically considered an insulating sheath surrounding axons to augment the speed of neuronal conduction. However, the fact that in demyelinating diseases, like Multiple Sclerosis (MS), the loss of myelin causes not only a slowdown of nerve impulses, but also a progressive axons degeneration, suggests that myelin plays a specific trophic role. The hypothesis rises that axonal degeneration occurs due to an energetic imbalance. Recently in vesicles of isolated myelin we reported the whole functional electron transport chain and ATP synthase [1]. Moreover, oxidative phosphorylation activity decreases in MS plaques proportionally to the severity of the lesions. A respiratory chain dysfunction could both depend on and cause the production of reactive oxygen species that, in damaging the membrane, may lead to a further loss of the sheath. By both biochemical and cytometric analysis we observed the effect of oxidative stress on oxygen consumption and ATP production in isolated myelin vesicles (IMV). This was possible because IMV react to typical mitochondrial fluorescent probes as JC1 or DHR and, due to their size, are visible to confocal microscopy as well as semiquantitative western blotting, oxymetric, lumimetric, and spectrophotometric analyses were performed to identified the respiratory chain in IMV and to observe oxygen consumption, ATP synthesis and respiratory complexes activity, respectively.

We observed that the presence of OXPHOS proteins in myelin increases parallely with the sheath development. Moreover, the oxygen consumption, ATP synthesis and respiratory complexes are functioning only after the 11th day after born and are similar to the control only in myelin isolated from rat at 33 days after the born.

These data advance the idea that the OXPHOS proteins play a role in the myelin sheath during its development and therefore the oxidative ATP production in myelin is not due to a mitochondrial contamination during the sample preparation. Moreover, the demonstration of the functioning OXPHOS in myelin could clarify the neuro-trophic role of myelin, giving a new input to the understand the correlation between the myelin loss and the axonal degeneration in demyelinating diseases.

**References**


**SW04.S19–12**

**Energetic metabolism of myelinated axons: a correlation among extramitochondrial ATP production in myelin and the sheath development**

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Brain functioning requires a lot of energy to restore and maintain its electro-physiological activity. It represents only 2–3% of the body weight, but its energy consumption is more than 20% of that of the whole organism. This is quite surprising because brain mitochondrial density is lower than in other organs with less energy demand. Several Authors suggest that part of the energy may be supplied by the glia [1]. Our attention has focused on myelin, the multilayered membrane protein/lipid structure surrounding axons.

Our recent study has demonstrated that the whole oxidative phosphorylation (OXPHOS) chain is present in isolated myelin; moreover, these proteins are able to produce ATP, consuming oxygen [2,3]. This suggests that myelin sheath, not only surrounds the axon, but supplies it, through energy production.

We have hypothesis that the OXPHOS proteins are transferred to myelin from the preformed inner mitochondrial membranes by the Golgi apparatus, as a consequence of a fusion among mitochondria and endoplasmic reticulum, during the sheath formation. So, considering that, in rat, the myelin sheath is formed only after the born, we have investigated the oxidative metabolism at several time-points of myelin development: 0, 5, 7, 11, 15, 33, post born days.

Experiments were conducted on isolated myelin vesicles (IMV), obtained according to the Norton and Poduslo method. The data are compared with a myelin isolated form an adult rat, used as a control. Both an imaging and a biochemical approach was utilized. Confocal microscopy as well as semiquantitative western blotting, oxymetric, lumimetric, and spectrophotometric analyses were performed to identified the respiratory chain in IMV and to observe oxygen consumption, ATP synthesis and respiratory complexes activity, respectively.

We observed that the presence of OXPHOS proteins in myelin increases parallely with the sheath development. Moreover, the oxygen consumption, ATP synthesis and respiratory complexes are functioning only after the 11th day after born and are similar to the control only in myelin isolated from rat at 33 days after the born.

These data advance the idea that the OXPHOS proteins are insert in the myelin sheath during its development and therefore the oxidative ATP production in myelin is not due to a mitochondrial contamination during the sample preparation. Moreover, the demonstration of the functioning OXPHOS in myelin could clarify the neuro-trophic role of myelin, giving a new input to the understand the correlation between the myelin loss and the axonal degeneration in demyelinating diseases.

**References**

values. By using RNAi-mediated knockdown we proved a crucial role of STIM1 protein in the SOC pathway in MSNs.

We suppose that SOC pathway is a novel target for HD treatment and EVP4593 is a perspective therapeutic agent for treatment of HD and possibly other polyglutamine diseases.

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SW04.S19–14
S100A4 calcium binding protein is differently expressed in patients with early-onset Parkinson’s disease associated with PINK1 W437X and PARK2 gene mutations

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Parkinson’s disease (PD) is a common neurodegenerative disorder; advanced age is the most important risk factor for sporadic PD, but six genes have conclusively been linked to PD [1]. Mutations in the Parkin gene at PARK2 locus are the most frequent known cause of early-onset (<40–50 years) PD (10–20% worldwide; about 50% of recessive familial forms, 15% of isolated cases in European populations) [2], on the contrary, homozygous and compound heterozygous loss-of-function mutations in PINK1 are the second most frequent cause of autosomal recessive early onset parkinsonism; the mutation frequency varies geographically from 0% to 15% worldwide [3]. We have analyzed the protein expression changes of primary fibroblast cultures from PINK1/W437X and PARK2ex 3-4 del familial PD patients by using integrated proteomic approaches. We found both patients down expressed S100A4 calcium binding protein with respect to control sample. This protein is a member of the S100 family that is composed by almost 25 members having the general property of calcium binding [4]. In fact we found an increase of intracellular calcium levels and a release of cytochrome c into the cytosol both in PINK1 and in Parkin mutant cells. As in the nervous system, high levels of S100A4 expression are observed at sites of neurogenesis and lesions, suggesting a role of the protein in neuronal plasticity [5], therefore we hypothesize the down expression of S100A4 could be the common mechanism by which PINK1 and Parkin promote excessive calcium accumulation disturbing mitochondrial calcium fluxes leading to neuronal cells death.

References

SW04.S19–15
Decreased protein arginine methylation in a mouse model of CBS deficiency

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Cystathionine beta synthase (CBS) deficiency is a rare disease characterized by high plasma levels of total homocysteine (tHcy) and a severe clinical outcome that often includes mental retardation. Hypomethylation due to build-up of the homocysteine precursor Sadenosylhomocysteine (AdoHcy), a potent methytransferase inhibitor, may underlie the etiology of CBS deficiency. We aimed at determining whether protein arginine methylation, a crucial post-translational modification that generates monomethylarginine (MMA) and dimethylarginine (asymmetric, ADMA, and symmetric, SDMA) residues, is disturbed in CBS deficiency.

We have used a mouse model in which the murine cbs gene has been deleted and animals express either the wild-type human variant (controls) or a mutant isoform (CBS deficient). S-adenosylmethionine (AdoMet) and AdoHcy levels in liver, heart, kidney and brain were measured by LC-EISI-MS/MS, and tHcy and protein-incorporated MMA, ADMA and SDMA were determined by HPLC with fluorimetric detection.

Mean tHcy levels in all tissues examined of the CBS deficient mice were at least 15-fold higher than in controls. Mean AdoMet levels were increased in liver (4.3-fold), kidney (1.5-fold) and brain (1.4-fold), while AdoHcy levels were more than sixfold increased in all tissues. Protein-incorporated ADMA was decreased in liver and brain (by 10% and 6%, respectively), as well as SDMA (by 9% and 35%, respectively), compared with controls.

We conclude that, in CBS deficient mice, the degree of protein arginine methylation is affected in a tissue-specific manner. Since arginine methylation modulates the function of several proteins, we reason that a loss of vital protein’s functionality due to hypomethylation may play a role in the pathophysiology of CBS deficiency. These results provide news insights and further research is running to disclose the role of SAH-mediated protein hypomethylation in hyperhomocysteinemia-associated vascular disease in humans.

SW04.S19–16
Normal versus mutant huntingtin: who wins the race?

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Protein misfolding and aggregation have been shown to be primarily responsible for the pathology of many neurodegenerative diseases. Solubilisation of such misfolded proteins has immense therapeutic benefits. Various small molecules can act as inhibitors of aggregation and have emerged as potential drug candidates. Huntington’s disease (HD) results from aggregation of mutant huntingtin protein (mhtt), bearing an elongated polyglutamine tract. The role of normal htt in the pathology of HD is ambiguous. Some reports indicate reduction of cytotoxicity in its presence while some studies report coaggregation of the two proteins. In this study, we have coexpressed normal and mutant htt in the well-established yeast model of HD. Our results show that the disease phenotype depends on the expression level of the normal protein. When expressed at a high level normal htt assists solubilisation of mhtt. Fluorescence Recovery after Photobleaching (FRAP) showed that solubilisation led to 97% mobile fraction as compared to only 15% mobility of mhtt in the absence of the normal protein. Our qFRET results conclusively show that there is direct interaction between normal and mutant htt. On the other hand, sequestration of normal protein occurred into mhtt aggregates when expression level of normal protein was low. Even in this case, the expression level of mhtt was higher than when it was expressed alone. The toxicity of mhtt is significantly reduced in yeast cells indicating that cell death in HD is due to gain of function of mhtt. This also answers the old controversy about the mechanism of cell death in HD. Our studies show that
equilibrium between folded and misfolded conformation of mhtt can be regulated by the presence of normal htt. The beneficial role of the latter, even when its expression is low, opens up an exciting route to inhibit aggregation of mhtt.

**SW04.S19-17**

NMR solution structure of rat beta amyloid metal-binding domain: new insights into the mechanisms of rats' resistance to Alzheimer's disease

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According to the amyloid cascade hypothesis the transition of amyloid-β (Aβ) monomer into neurotoxic dimmers, oligomers, and fibrillar aggregates is a key event in Alzheimer’s disease (AD) pathogenesis. Zinc ions play a critical role in pathological dimerization of Aβ metal-binding domain. In contrast to other mammals, rats and mice are invulnerable to AD probably due to three amino acid substitutions (R5G, Y10F, and H13R) in Aβ metal-binding domain. In an attempt to reveal the mechanism of rats' resistance to AD, we obtained the structure of the metal-binding domain 1–16 of rat β-amyloid (rat Aβ[1–16]) in solution in the absence and presence of zinc ions. A zinc-induced dimerization of the domain was detected. The zinc coordination site was found to involve residues His-6 and His-14 of both peptide chains. We used experimental restraints obtained from analyses of NMR and isothermal titration calorimetry data to perform structure calculations. The calculations employed an explicit water environment and a simulated annealing molecular-dynamics protocol followed by quantum-mechanical/molecular-mechanical optimization. We revealed that the C-tails of the two polypeptide chains of the rat Aβ[1–16] dimer are oriented in opposite directions to each other, which hinders the assembly of rat Aβ dimers into oligomeric aggregates. Thus, the differences in the structure of zinc-binding sites of human and rat Aβ[1–16], in their ability to form regular cross-monomer interactions, and in the orientation of the hydrophobic C-tails could be responsible for the rats’ resistance to AD.

**SW04.S19-18**

Type 1 diabetes affects expression of 14-3-3 proteins in a tissue specific way

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Type-1 diabetes (T1D) is an endocrine disorder caused by autoimmune destruction of pancreatic β cells, leading to uncontrolled chronic hyperglycemia. Complications related to T1D result in the dysregulation of several cellular pathways leading to target organ damages like diabetic cardiomyopathy, hepatic and renal failure and diabetic encephalopathy. A most used animal model of T1D is obtained by the administration of streptozotocin (STZ) to rats, thus giving the opportunity to tightly monitor the onset of the disease. The 14-3-3 family proteins are key signaling molecules, involved in the regulation of multiple cellular pathways, mainly the apoptotic one, consisting of seven distinct isoforms (β, γ, ε, ζ, τ, η, σ), mainly localized in the cytosol.

In this study we investigated the expression of 14-3-3 family proteins at transcript and protein levels in the brain and liver of short-term T1D rats.

T1D was induced by STZ treatment and after 3-weeks purified brain and liver cytosolic proteins were prepared from control and T1D rats. The protein expression of all the seven 14-3-3 isoforms were studied by Western blot analysis using high-specific monoclonal antibody and the relative transcript levels were assessed by Real-time quantitative PCR. At brain level all the 14-3-3 isoforms showed a statistical significant changes in protein expression and mRNA level in T1D sample compared to control ones. In detail, six of the seven 14-3-3 isoforms (β, γ, ε, ζ, τ, η) showed a marked decrease of both protein and mRNA content in the T1D brain, while for the 14-3-3 ε isofrom the protein decreased level was opposite to the increased mRNA content in T1D rats. This finding could be attributed to post-translational events or to an enhanced binding of the protein with one of its multiple targets.

On the other hand, in the liver of T1D rats only one 14-3-3 isoform (γ) showed a slight statistical significant change, showing in this case an increment in both protein content and mRNA level. Overall our results indicate that the impact of short-term T1D on 14-3-3 proteins and transcript expression is different at brain and hepatic level. This might be associated with activation or inactivation of apoptotic pathways in a tissue-specific way.

**SW04.S19-19**

The combination of Nerve Growth Factor (NGF) with valproic acid and Trichostatin A increases apoptosis via NGF receptor p75NTR and suppresses proliferation of C6 glioma cells

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Removal of acetyl moieties from histones and some proteins is used widely as a therapeutic aim in cancer and neurodegenerative diseases. Histone deacetylase inhibitors stimulate the hyperacetylation in chromatin, which causes activation of some genes and it also stimulates the terminal cell differentiation and/or apoptosis. Valproic acid (VPA) is an agent whose antineoplastic and neuroprotective features are known and it can change the expression of several targets including neurotrophines in central nervous system. Trichostatin A (TSA) inhibits cellular cycle in eukaryotic cells and it can change gene expression. It is known that rat C6 glioma cells express nerve growth factor (NGF) receptors both in vitro and in vivo, and they also cause differentiation of these cells, leading to inhibition of their proliferation. This study was designed to determine the effects of two histone deacetylase inhibitors on especially apoptosis and cellular proliferation mechanism in C6 glioma cells and relationship to the administration of NGF. By considering all these data, VPA, TSA and NGF administered in different doses (VPA: 0.1, 0.5, 1, 3, 5, 10 mM, TSA: 5, 10, 25, 50, 100, 200 ng/ml, NGF: 10, 50, 100 and 200 ng/ml) and periods (24 and 48 hours) individually (VPA, TSA and NGF) and in combined manner (VPA+TSA, VPA+TSA+NGF). The NGF levels, cell proliferation and cell death were determined with colormetric (ELISA) manner, whereas caspase-3 activity, p75NTR and TrkA determined with immunocytocchemical manner. We reported that VPA, TSA and NGF (individually and combined manner) inhibited the cell proliferation and triggered apoptosis in C6 glioma cells. In the present study, it is suggested that apoptotic cell death triggered by NGF is mediated via p75NTR. By considering these data, we suggest that VPA, TSA and NGF combination may be new therapeutic agents in the therapy of neurodegenerative diseases and glial derivative brain cancer.
**SW04.S19–20**

**Melatonin’s effect on inflammatory cytokines in oxidative stress induced PC12 cells**

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**Introduction:** Melatonin is primarily synthesized and secreted by the pineal gland. Melatonin participate in many physiological and biochemical functions. The etiology of neurodegenerative diseases has not been well understood. Oxidative stress and inflammation seem like attributed to generation of these diseases. The increase in oxidative stress is one of the markers of inflammation in the body. Brain is a tissue highly vulnerable to oxidative stress. Melatonin is known as an antioxidant beside its anticancerogen and antia apoptotic effects. Melatonin is being related to many neurodegenerative disease but its mechanism hasn’t been understood yet. In this study our aim is to evaluate the antiinflammatory effect of melatonin on oxidative stress induced PC12 cells.

**Method:** We used PC12 cells in the study. Cells were divided into 75 cm² cell flask, the last volume was 10 ml in each flask. Culture flasks was marked into four groups which one of them was control group, the other was treated with melatonin and H2O2 at chosen doses as below: Groups are: (i) Control, (ii) Melatonin (5 × 10⁻⁷), (iii) H2O2=Melatonin (5 × 10⁻⁷), 4. H2O2

All plates were incubated for 72 hours at 37°C incubator. After incubation, protein extraction was performed with Trizol and Western Blotting was performed for the evaluation of IL-2, IL-6, TNF-α and NFκB.

**Results and Conclusion:** We found oxidative stress induced PC12 cells revealed higher levels of inflammation factors however treatment of the cells with melatonin for 72 saat decreased the levels of these cytokines with the level of control cells. We can see the protective effect of melatonin on inflammatory cytokines in oxidative stress conditions.

**SW04.S19–21**

**Alzheimer disease hallmarks in adult type 2 diabetic female rat brain: Is oxidative stress the predecessor?**

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Diabetes mellitus is one of the most common heterogeneous metabolic diseases and is characterized by an uncontrolled chronic hyperglycemia and an insulin dysfunction. The increased prevalence of type 2 diabetes (T2D), the association with aging and lifestyle, the complications in several organs including the brain and the multiple co-relations with the development of neurodegenerative disorders are some of the aspects responsible for the increased attention to this type of diabetes. Numerous studies have shown that people with T2D have twice the incidence of sporadic Alzheimer disease (AD), being insulin signaling abnormalities considered one of the main links between the two pathologies. Besides that, the prevalence of AD and other dementias seem higher in women than in men, and some studies show controversies in whether diabetic female are more or less prone to cognitive dysfunction than males. In this regard, as there are hormone signaling cascades that may converge with the insulin receptor (IR)/insulin growth factor (IGF-1)-receptor signaling, we hypothesized that gender affects IR/IGF-1-mediated intracellular signaling pathways and AD-like pathological hallmarks in adult T2D female rat brains. Herewith, we analyzed brain cortical homogenates from adult (8-months-old) female Wistar control and T2D Goto-Kakizaki (GK) rats. GK rats are characterized by higher blood glucose levels, as we also observed in this study. In addition, brain cortical homogenates from GK female rats presented higher cytosolic cholesterol and dehydroepiandrosterone (DHEA) levels, even though testosterone and estrogen levels were lower, suggesting that this may reflect a dysfunction in steroid hormone metabolism. Furthermore, an increase in insulin and IGF-1 levels occurred, which was not accompanied by changes in IR and IGF-1R protein expression levels, suggesting a situation of insulin and IGF-1 resistance in diabetic rats. Accordingly, GK females also showed lower P-Akt and P-ERK1/2 protein expression levels. Interestingly, these females’ brains displayed an increased oxidative stress, seen through the increase in the levels of thiobarbituric acid reactive substances (TBARS). Despite these alterations, amyloid beta peptide and hyperphosphorylated tau protein levels (AD features) remained unchanged in GK females. To summarize, these results imply that on one hand the oxidative damage occurring in adult T2D female rat brain may be triggered by the dysfunctional steroid hormone metabolism and the insulin and IGF-1 resistance, and on the other hand that it might be the predecessor for the development of AD hallmarks.


**SW04.S19–22**

**Familial Alzheimer’s disease PS1 gene mutants affect activity of calcium channels differently**

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Familial Alzheimer’s disease (FAD) is caused by mutations in presenilin-1 (PS1) gene in approximately 50% of cases. PS1 is well known as a component of the gamma-secretase enzyme which cleaves APP to A-beta and Notch. To become a catalytic part of enzyme PS1 holoprotein undergoes an androteolysis. It was shown that FAD PS1 mutants disrupt calcium homeostasis in hippocampal neurons. In our study we found different effects of three FAD PS1 mutants (PS1 M146V, PS1DE9, PS1 D247A) on ER calcium storage and activity of store-operated and voltage-gated calcium channels in neurons and human neuroblastoma SK-N-SH cell line. Disrupted channels activities were
detected with direct single-cell electrophysiological measurements. Intracellular calcium concentrations were measured in calcium imaging experiments with fur2-AM. The different effects were connected with calcium sensors STIM1 or STIM2 impaired signal transduction from ER to calcium channels in plasmatic membrane (PM) under control of ER calcium levels. The impaired signal transduction was revealed in live confocal imaging experiments. All the effects were caused by PS1 holoprotein. The PS1 endoproteolysis levels were estimated with SDS PAGE and western-blot of total cell lysates.

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SW04.S19–23
Cytochrome P450 enzymes specific expression and modulation in brain as a tool to treat bilirubin encephalopathy

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Bilirubin encephalopathy, is caused by the deposition of unconjugated bilirubin (UCB) in the brain leading to a damage at specific structures (hippocampus and cerebellum more damaged) [1].

When bilirubin conjugation is impaired in liver, UCB accumulation may be oxidize by alternative mechanisms of the hepatic cytochromes P450 enzymes (Cyp1A1, Cyp1A2 and Cyp2A3; Cyps) [2,3]. Since the role of Brain Cyps (bCyps) in bilirubin clearance is unknown, we used astrocytes primary cultures from cortex (Cx: not damaged) and cerebellum (Cl: damaged) to assess: A) their inducibility by UCB exposure and B) induction of bCyps by β-naphthoflavone; βNF, to increase brain UCB clearance preventing UCB neurotoxicity.

Bilirubin was able to induce bCyps, both at the mRNA and functional (EROD; MROD) level. Of notice, Cyps (1A1 and 1A2) in astrocytes derived from Cx were more inducible that those from Cl.

The mRNA bCyps in astrocytes from Cx and Cl exposed to βNF was different both in timing and extent. Cyp1A1 maximal induction (eightfold) in Cx was reached at 6H, while in Cl (12-fold) at 24H. Cyp1A2 induction was threefold higher in Cx than Cl. Cyp2A3 was slightly induced in both Cx and Cl. The activity was induced similarly to mRNA expression.

These data define the dynamics of Cyp1A1, 1A2 and 2A3 selective induction in different brain regions with different susceptibility to UCB damage, and establish a concrete background for using bCyps to increase local brain bilirubin clearance to limit the damage.

References

SW04.S19–24
Repetitive mild traumatic brain injury in early life after blood-brain barrier integrity in immature rats

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In this study, the effects of an experimental method for the study of repetitive mild traumatic brain injury (TBI) on blood-brain barrier (BBB) integrity were investigated in immature rats. To evaluate BBB integrity, Evans blue (EB) and horseradish peroxidase (HRP) tracers were used. Tissue expression of occludin and aquaporin (AQP)-4, tissue activities of IL-1alpha IL-1beta, TNF-alpha and the levels malondialdehyde and glutathione were determined. Immunohistochemistry was studied using occludin and AQP-4 antibodies. No mortality was observed in immature rats after even the last induction of repetitive mild TBI. IL-1beta and TNF-alpha activities increased significantly (p < 0.01) in cerebral cortex of repetitive mild TBI group (p < 0.05). AQP-4, IL-1alpha and occludin activities in cerebral cortex and hippocampus did not show any differences among groups. The levels of glutathione and malondialdehyde increased significantly in cerebral cortex of repetitive mild TBI group compared to sham (p < 0.05). The immunoactivity of occludin hippocampus and AQP-4 in parietal cortex remained relatively intact in repetitive mild TBI group. EB extravasation into brain did not show significant differences between groups, however ultrastructurally frequent vesicles containing HRP reaction products were detected in the cytoplasm of capillary endothelial cells in cerebral cortex and hippocampus in repetitive mild TBI group. These results suggest that repetitive mild TBI cause the limited transfer of circulatory substances into brain parenchyma molecular weight <40 kDa and the impaired BBB which could cause by IL-1beta, TNF-alpha or MDA subsequent repetitive mild TBI in immature animals.

SW04.S19–25
Dysfunction of neuromuscular synapses in transgenic mice with Alzheimer’s disease model

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Alzheimer’s disease (AD) is incurable neurodegenerative disorder which is characterized by progressive cognitive decline. Numerous data show that synaptic pathology can be central for AD pathogenesis; however, mechanisms of synaptic dysfunction in AD are not well understood. Here we studied neuromuscular synaptic transmission and skeletal muscle fiber electrogensis in AD transgenic mice.

Experiments were performed on diaphragmatic preparations of AD transgenic mice (B6C3-Tg(APP695)85Db Tg(PSEN1)85Db genotype (APP–PSEN1 mice) and wild type...
SW04. S19–26
Inhibition of neutral sphingomyelinase decreases elevated levels of nitrotyrosine and inducible nitric oxide synthase in ocular hypertensive rats
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The aim of this study was to determine the role of selective neutral sphingomyelinase (N-SMase) inhibition on inducible nitric oxide synthase (NOS-2) levels and retinal physiological function in a rat model of elevated intraocular pressure (IOP). Rats were randomly divided into different experimental groups which received either an intraperitoneal N-SMase inhibitor (GW4869; 1.25 mg/kg/day) or vehicle (DMSO) for a period of 6 weeks. Ocular hypertension was induced by unilaterally cauterizing three episcleral vessels and the unoperated eye served as control. Levels of NOS-2 and nitrotyrosine in retinal tissue were determined by immunohistochemical analysis and ELISA, respectively. Retina N-SMase activity and nitrite/nitrate levels were measured by fluorimetric method while neuroprotective effect of GW4869 was determined via electrophysiological measurements of visual evoked potentials (VEP). Elevated IOP did not cause a significant increase in retina N-SMase activity compared to control groups while administration of GW4869 significantly reduced N-SMase activity in retinal tissue. Latencies of all VEP components (P1, N1, P2, N2, P3) were significantly prolonged in EIOP and returned to control levels following GW4869 treatment. Immunostaining of NOS-2 and nitrate/nitrite levels were significantly greater in non-treated rats with EIOP compared to GW4869 treated rats. Ocular hypertension significantly increased retinal protein nitration which returned to baseline levels following GW4869 treatment. The presented data confirm the role of nitrative injury in elevated IOP and highlight the protective effect of N-SMase inhibition in ocular hypertension via downregulation of NOS-2 levels and nitrative stress. Acknowledgement: This study was supported by a grant (111S419) from TUBITAK.

SW04. S19–27
The role of oxidative/antioxidative balance, vascular pathophysiology and inflammation in migraine
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Migraine is classified as a primary headache according to the classification devised by International Headache Society. It is generally defined as a neurovascular disorder characterized by neurologic, gastrointestinal and autonomous changes, neurogenic inflammation, contractile dysfunction of cerebral vascularity and depressive mechanisms originating from cerebral cortex and propagating throughout the brain. Various studies focused on oxidative stress, neuroinflammatory conditions, cytokines, several neuropeptins and numerous vasomotor changes in migraine patients. Free radicals are highly reactive molecules formed during the transformation of nutrients to energy using oxygen. They are generally too reactive and have a half-life too short and attack different molecules in cells, tissues or body fluids. Free radicals can cause severe damage to the normal cells of the body. This damage can be to the lipids, DNA, proteins, carbohydrate and other macromolecules and forms the basis of a wide variety of diseases such as atherosclerosis, cardiovascular disease, cancer, cerebrovascular disorders, neurodegenerative problems, diabetes, acute renal failure, lung disease and alcoholic liver disease. Eukaryotic cells produce oxidants during normal metabolic events such as respiration and phagocytosis and in response to growth factors and cytokines. In aerobic organisms, antioxidant mechanisms have evolved to regulate free radical production and stall their harmful intracellular effects. Oxidative stress occurs under pathophysiological circumstances when the amount of oxidants exceeds the scavenging capacity of cellular antioxidant systems. In this study, we have examined the contribution and roles of different factors such as oxidative stress, vascular pathophysiology and inflammation to the pathogenesis of migraine disease. Antioxidant capacity of migraineurs were significantly lower than controls, whereas total oxidant status was higher in patients compared to the controls. This preliminary study showed that oxidative/antioxidative balance shifted towards the oxidative status in migraine. A neuroimmune mechanism for migraine was tested with expected increases in some cytokines tested during a migraine. Changes of the level of cytokines in the blood of patients with migraine may suggest that neurogenic inflammation participates in the pathogenesis of migraine. These results may suggest different roles of antioxidant/oxidant balance, neuroimmune status and vascular pathophysiological markers in migraine pathogenesis which may be applied to treatment protocols in the future.

SW04. S19–28
Expression profile of genes associated with oxidative/antioxidative events during the development of age-related macular degeneration in OXYS rats
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Age-related macular degeneration (AMD), the leading cause of vision loss in the elderly, is a complex and heterogeneous disease.
that involves the interaction of both genetic and environmental factors. Recently Nuclear factor erythroid 2-related factor 2 (Nrf2) and aryl hydrocarbon receptor (AhR) were considered as AMD candidate genes. Transcription products of the AhR-Nrf2 ‘gene battery’ are important in mediating the cellular response to the oxidative stress. To assess the nonspecific action of Nrf2-signaling pathway on the development of AMD, we compared transcriptional activity of Nrf2 and it regulates genes in the retina of senescence-accelerated OXYS rats and avowed model of AMD, of OXYSb rats with tardy retinopathy development and Wistar rats without signs of disease. mRNA levels of AhR, AhR-regulated (CYP1A1, CYP1A2, CYP1B1, ALDH3A1, UGT1A9), Nrf2, Nrf2-regulated (Hmox1, Txnrd1, Gsr) and genes regulated by both AhR and Nrf2 (GSTA1, NQO1, UGT1A6) was determined in the age of 1, 3 and 12 month (mo) by qRT-PCR. Study showed that at the age of 1 month, when the rats are no signs of the AMD, mRNAs of AhR and Hmox1 genes in the retina of OXYS were lower as compared to Wistar rats while mRNA of Nrf2 and Gsr were higher as compared to OXYSb rats. At the age of 3 months, when OXYS rats revealed signs of AMD mRNAs of CYP1A1, CYP1A2, UGT1A6 and NQO1 in OXYS rats were decrease compared with Wistar and mRNAs of CYP1A2 and NQO1 compared with OXYSb. Expression of GSTA1, Txnrd1 and Gsr genes in OXYSb rats was higher than in Wistar. At the age of 12 months, when develop forms of retinopathy in OXYS rats and manifestation of early stage of disease in OXYSb are observed, only expression of the CYP1B1 was lower and the Hmox1 higher in the retina of OXYSb rats as compared to OXYS. Identified specifics of the expression profile of genes functionally associated with both pathways of antioxidant defense and with the development of oxidative stress may contribute to pathogenesis of AMD in OXYS rats. One of the triggers for the onset of oxidative stress may be the inborn reduced level of AhR expression in OXYS rats. The higher expression levels of antioxidant genes Nrf2 and Gsr in 1-mo-old OXYSb rats may explain a later onset and less severe progression of AMD in rats OXYSb compared to OXYS.

**SW04.S19–29**

**Laboratory diagnostics of muscle-specific receptor tyrosine kinase auto-antibodies in patients with myasthenia gravis without acetylcholine receptor antibodies**

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Myasthenia gravis (MG) is a disease caused by disordered neuromuscular transmission on neuromuscular plate. Auto-antibodies to postsynaptic acetylcholine receptors (AChR) are detectable in the serum of 80–90% of patients with generalized MG. Muscle-specific receptor tyrosine kinase auto-antibodies (AMUSK) in patients with myasthenia gravis without acetylcholine receptor antibodies are found in 7% of cases.

We would like to evaluate the proportion of muscle-specific receptor tyrosine kinase auto-antibodies in patients with myasthenia gravis without acetylcholine receptor antibodies.

We have tested acetylcholine receptor antibodies with kits ARAb RRA, IBL and muscle-specific receptor tyrosine kinase auto-antibodies with kits MUSK RRA, DLD. In both cases we used quantitative radioreceptor immunological estimation of antibodies in serum.

Since the year 2000 we have tested 3810 of patients with diagnosis MG. Negative levels of AChR in 66% patients with MG oscillate under 0.4 nmol.l⁻¹. The values ranged from 0.4 to 8.0 nM in 1% of patients and we detected values over 8 nM in 33% of patients. AMUSK were later measured in 386 patients, whose negative levels of AChR were lower then 0.4 nM. Positive AMUSK (>0.03 nM) was found in 5% of AChR negative patients.

Myasthenia gravis is the second most frequent neurological autoimmune disease. At first the levels of antibodies against AChR are measured, which are the main cause of MG. Considering the fact, that AMUSK can cause the nerve-muscle disorders in patients with negative values of AChR, is to eliminate their ratio as source of disorder of postsynaptic nerve-muscle transfer. Measurement of antibodies against AChR and AMUSK is useful for diagnostic process of MG.

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**SW04.S19–30**

**Neurosteroids and cholesterol catabolism by steroid 7alpha-hydroxylases**

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The classical and quantitatively most important pathway of cholesterol catabolism in humans is bile acid biosynthesis. Several enzymes are involved in this pathway, among them cytochromes P450, notably members of CYP7 family: cholesterol 7α-hydroxylase and oxysterol 7α-hydroxylase. While cholesterol 7α-hydroxylase (CYP7A1) catalyses first and rate limiting step in classic pathway, oxysterol 7α-hydroxylase (CYP7B1) involved in alternative pathway. However, the role of CYP7B1 seems to be more important in metabolism of sex hormone precursors and neuroactive steroids as mutations of CYP7B1 gene cause spastic paraplegia-5A (SPG5A), motor-neuron degenerative disease. To understand different substrate specificities of two 7α-hydroxylases we overexpressed, purified and characterize recombinant CYP7B1 in comparison with CYP7A1. Our data confirm that typical substrates of CYP7B1 (25-hydroxycholesterol, 27-hydroxycholesterol, pregnenolone, DHEA, 5α-androstane-3β, 17β-diol) show only weak or no binding to CYP7A1. However, the principal difference between 7A1 and 7B1 is in metabolism C19- and C21-steroids. CYP7A1 cannot metabolize these steroids, whereas CYP7B1 has specific structural requirements for the sterol ring system. To gain further insight into the structural basis of substrate recognition, we determined crystal structures of CYP7A1 alone and in complex with the substrate cholest-4-en-3-one and the inhibitor 25-hydroxycholesterol. 27-hydroxycholesterol, pregnenolone, DHEA, 5α-androstane-3β, 17β-diol show only weak or no binding to CYP7A1. However, the principal difference between 7A1 and 7B1 is in metabolism C19- and C21-steroids. CYP7A1 cannot metabolize these steroids, whereas CYP7B1 has specific structural requirements for the sterol ring system. To gain further insight into the structural basis of substrate recognition, we determined crystal structures of CYP7A1 alone and in complex with the substrate cholest-4-en-3-one and the inhibitor 7-ketocholesterol. Cholesterol oxidation products, especially 7-ketocholesterol are highly toxic and present in abundance in atherosclerotic plaques. 7-ketocholesterol have been the focus of much attention as they cause the disease. The other mutations in CYP7A1 gene are being studied. The classical pathway of cholesterol catabolism is involved in metabolism of sex hormone precursors and neuroactive steroids as mutations of CYP7B1 gene cause spastic paraplegia-5A (SPG5A), motor-neuron degenerative disease. To understand different substrate specificities of two 7α-hydroxylases we overexpressed, purified and characterize recombinant CYP7B1 in comparison with CYP7A1. Our data confirm that typical substrates of CYP7B1 (25-hydroxycholesterol, 27-hydroxycholesterol, pregnenolone, DHEA, 5α-androstane-3β, 17β-diol) show only weak or no binding to CYP7A1. However, the principal difference between 7A1 and 7B1 is in metabolism C19- and C21-steroids. CYP7A1 cannot metabolize these steroids, whereas CYP7B1 has specific structural requirements for the sterol ring system. To gain further insight into the structural basis of substrate recognition, we determined crystal structures of CYP7A1 alone and in complex with the substrate cholest-4-en-3-one and the inhibitor 7-ketocholesterol. Cholesterol oxidation products, especially 7-ketocholesterol are highly toxic and present in abundance in atherosclerotic plaques. 7-ketocholesterol is a specific CYP7A1 inhibitor and recently was shown to be the product of CYP7A1 action on 7-dehydrocholesterol. Binding mode of different steroids in CYP7A1 active site provides a template for CYP7B1 and enabled us to model and test several mutations found in patients with SPG5A. One of the mutants R487C in the vicinity of the CYP7A1 active site provides a template for CYP7B1 and enabled us to model and test several mutations found in patients with SPG5A. One of the mutants R487C in the vicinity of the CYP7A1 active site provides a template for CYP7B1 and enabled us to model and test several mutations found in patients with SPG5A. One of the mutants R487C in the vicinity of the CYP7A1 active site provides a template for CYP7B1 and enabled us to model and test several mutations found in patients with SPG5A.
**SW04.S19–31**

**Knockdown of GCAP1 rescues dominant retinal degeneration**

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Dominant mutations occurring in the high-affinity Ca$^{2+}$-binding sites (EF-hands) of the *GUCA1A* gene encoding guanylate cyclase-activating protein 1 (GCAP1) cause slowly progressing cone-rod dystrophy (CORD) in a dozen families worldwide. We developed a nonallele-specific adeno-associated virus (AAV)-based RNAi knockdown strategy to rescue the retina degeneration caused by GCAP1 mutations. We generated three genomic transgenic mouse lines expressing wildtype (WT) and L151F mutant mouse GCAP1 with or without a C-terminal GFP fusion. Under control of endogenous regulatory elements, the transgenes were expressed specifically in mouse photoreceptors. GCAP1(L151F) and GCAP1(L151F)-GFP transgenic mice presented with a late onset and slowly progressive photoreceptor degeneration, similar to that observed in human GCAP1-CORD patients. Transgenic expression of WT GCAP1-EGFP in photoreceptors had no adverse effect. Toward therapy development, a highly effective anti-mGCAP1 shRNA, mG1hp4, was selected from four candidate shRNAs using an *in vitro* screening assay. Subsequently a self-complementary (sc) AAV serotype 2/8 expressing mG1hp4 was delivered subretinally to GCAP1(L151F)-GFP transgenic mice. Knockdown of the GCAP1(L151F)-GFP transgene product was visualized by fluorescence live imaging in the scAAV2/8-mG1hp4-treated retinas. Concomitant with the mutant GCAP1-GFP fusion protein, endogenous GCAP1 decreased as well in treated retinas. We propose nonallele-specific RNAi knockdown of GCAP1 as a general therapeutic strategy to rescue any GCAP1-based dominant cone-rod dystrophy in human patients.

**SW04.S19–32**

**Yeast models for mammalian protein aggregation disorders**

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Self-perpetuating ordered cross-beta protein aggregates (amyloids) are associated with a variety of mammalian and human neurodegenerative disorders, including Huntington’s disease (caused by polyglutamine expansion), prion diseases (caused by PrP protein) and Alzheimer’s disease (caused by Abeta peptide). We employed a yeast assay for studying the aggregation and toxicity of polyglutamines. In mammalian cells, large cytoplasmic deposits of aggregated polyglutamines (aggresomes) are thought to be cytoprotective rather than cytotoxic. Our data (Gong et al. 2012 PLoS Genetics 8: e1002634) show that in yeast, one and the same type of aggresome-like polyglutamine deposit could be either cytotoxic or cytoprotective, depending on the composition of endogenous protein aggregates (yeast prions) present in the cell. Moreover, endogenous prions mediate sequestration of other proteins by polyglutamines, while overproduction of a sequenced protein ameliorates cytotoxicity. These data explain some discrepancies of previous studies and shed light on the potential nature of ‘non-genetic’ factors regulating the onset of Huntington’s disease in humans. We also demonstrate that fusions of mammalian amyloidogenic proteins (including PrP and Abeta) to the prion-forming domain of a yeast prion promote de novo nucleation of the yeast prion in the absence of any pre-existing prions. Alterations of PrP or Abeta, abolishing their prionogenic or amyloidogenic properties in mammalian or in vitro systems, also eliminate prion nucleation in yeast. In contrast, truncated PrP derivatives associated with a heritable prion disease in humans exhibit significantly increased prion nucleation properties in yeast. The 42 amino acid derivative of Abeta (Abeta42), having a higher aggregation propensity, exhibits much higher prion nucleation efficiency, compared to the 40 amino acid derivative (Abeta40). Our data establish a yeast-based assay for studying amyloidogenic properties of mammalian proteins involved in protein assembly disorders.

**SW04.S19–33**

**Involvement of hippocampal neuropeptide Y system in methamphetamine-induced memory impairment: the role of NPY Y2 receptors**

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Methamphetamine (METH) is a very addictive psychostimulant drug that causes irreversible brain damage leading to several neurological and psychiatric abnormalities, including cognitive deficits. Neuropeptide Y (NPY) is abundant in the mammalian central nervous system (CNS) and has several important functions, including the modulation of learning and memory processes. It has been demonstrated that METH induces significant alterations in mice striatal NPY, Y1 and Y2 receptor mRNA levels. However, the impact of this drug on the hippocampal NPY system and its consequences remain unknown. In the present study, we investigated the effect of METH intoxication on mouse hippocampal NPY levels, NPY receptors function, and memory performance. Results show that METH increased NPY, Y2 and Y5 receptor mRNA levels, as well as total NPY binding accounted by opposite up- and down-regulation of Y2 and Y1 functional binding, respectively. Moreover, both memory impairment and activation of AKT/mammalian target of rapamycin (mTOR) pathway induced by METH were prevented by the pharmacological blockade of Y2 receptors. These findings demonstrate that METH interferes with the hippocampal NPY system, particularly the Y2 receptors, which seem to be associated with memory deficits triggered by METH intoxication.

**SW04.S19–34**

**Diabetes alters KIF1A and KIF5B motor proteins in the hippocampus**

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Diabetes mellitus is the most common metabolic disorder in humans. Diabetic encephalopathy is a frequent complication of this pathology that leads to cognitive and memory impairments, which have been associated with structural and functional changes in the hippocampus. However, the mechanisms underlying...
ing those impairments triggered by diabetes in the hippocampus are far from being clarified.

In this study, we investigated the effect of diabetes (2 and 8 weeks duration) on motor proteins important for anterograde axonal transport, namely KIF1A (kinase that transports synaptic vesicle precursors containing synaptophysin and synaptotagmin) and KIF5B (kinase that transports mitochondria and membrane organelles containing proteins such as syntaxin-1 and SNAP-25), in the hippocampus.

The content of those proteins was evaluated by immunohistochemistry in hippocampal slices and by immunoblotting in total hippocampal extracts from streptozotocin-induced diabetic and age-matched control Wistar rats. Diabetes increased the immunoreactivity of KIF1A and KIF5B in the hippocampus. Since hyperglycemia is considered a major player in diabetic complications, the effect of a prolonged exposure to high glucose on motor proteins, mitochondria and synaptic proteins in hippocampal neurons was also studied, giving particular attention to changes occurring in axons. Hippocampal cell cultures were cultured for 14 days and were exposed to high glucose (50 mM) or mannitol (osmotic control; 25 plus 25 mM glucose), for 7 days.

In hippocampal cultures incubated with high glucose we did not detect changes in the intensity of fluorescence or number of accumulations related with mitochondria in axons of hippocampal neurons. Nevertheless, high glucose induced an increase in the number of fluorescent accumulations of KIF1A and synaptotagmin-1 and decreased KIF5B, SNAP-25 and synaptophysin immunoreactivity specifically in axons of hippocampal neurons. These changes suggest that anterograde axonal transport may be impaired in hippocampal neurons, which may lead to changes in the content of synaptic proteins in nerve terminals, thus contributing to previously detected alterations in neurotransmission in the hippocampus and cognitive and memory impairments in diabetic humans and animal models.

**SW04.S19–35**
The suppressive effect of IL-27 on encephalitogenic Th17 cells induced by multiwalled carbon nanotubes reduces the severity of autoimmune experimental encephalomyelitis

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**Background:** Both Th1 and Th17 CD4⁺ T lymphocytes specific for neuroantigens are described as encephalitogenic in the experimental autoimmune encephalomyelitis (EAE), which is a model of studying multiple sclerosis.

**Aim:** To investigate how multiwall carbon nanotubes (MWCNTs) internalized by antigen-presenting cells (APC) affect the development of encephalitogenic CD4⁺ T cells.

**Methods:** Encephalitogenic CD4⁺ T cells lines were stimulated in the presence or not of MWCNTs. The profile of the encephalitogenic T cells and their capacity to induce EAE were evaluated by adoptive transfer.

**Results:** APCs that internalized carbon nanoparticles were activated and produced significant amount of IL-27. Encephalitogenic CD4⁺ T cells cultured with APCs previously incubated with MWCNTs do not express IL-17 or express RORγt. The adoptive transfer of these cells causes less severe EAE. Moreover, the incubation of encephalitogenic T cells devoid of Th17 cells with neutralizing anti-IL-27 antibodies restored the production of IL-17. These results suggest that the increased IL-27 level produced by the APCs incubated with the carbon nanotubes inhibits the development of Th17 cells.

**Conclusion:** The results presented suggested that the stimulation of APCs with carbon nanoparticles prior to neuroantigen presentation affects the development of the Th17 subset of encephalitogenic CD4⁺ T lymphocytes and results in less severe EAE.

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**SW04.S19–36**
Single particle tracking reveals that amyloid aggregates alter the mobility of GM1 ganglioside on the plasma membrane of living cells

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Neuronal impairment in Alzheimer’s disease (AD) is currently attributed to a complex cascade of events triggered by the interaction of amyloid oligomers, constituted primarily by Abeta1-42 peptide, with the plasma membrane. Amongst the variety of toxic mechanisms proposed, one involves the binding of amyloid species to GM1 gangliosides. GM1 takes part into the formation of membrane rafts, dynamic and specialized membrane microdomains responsible for the compartmentalization of cellular processes such as signalling and protein trafficking. The interaction with GM1 has been demonstrated to be a crucial factor also in mediating the aggregation and toxicity of other amyloidogenic proteins and peptides, such as amylin (also known as human islet polypeptide, hIAPP), whose aggregation is associated to the development of type II diabetes. In particular, GM1 is fundamental in mediating the binding of preformed Abeta oligomers and amylin aggregates to synthetic lipid vesicles and causing their subsequent permeabilization. Exogenously applied oligomeric Abeta1-42 has been shown to accumulate on the membrane of cultured neurons at the level of rafts enriched in GM1. Nevertheless, experiments providing compelling evidence of interaction between Abeta oligomers and GM1 in living cells are still missing. The consistent, although sometimes conflicting, body of literature on the interaction of GM1 with amyloid species relies on averaged results obtained using bulk methods. In this case, many important details can be missed and only the most prominent features are eventually taken into account.

Here we take advantage of single particle tracking (SPT) techniques to monitor in real-time in living cells the dynamics of GM1 following the binding of amyloid aggregates of Abeta1-42 and amylin to the plasma membrane. We demonstrate that a direct interaction takes place in vivo, heavily affecting the diffusion properties of a subpopulation of GM1 molecules. Our results might imply an additional mechanism of toxicity, where amyloid aggregates alter cellular processes dependent on membrane raft mobility and clustering.
**SW04.S19–37**

Intercellular and intracellular signaling modulate PDT-induced death of neurons and glial cells

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Photodynamic therapy effect (PDT) is based on generation of singlet oxygen, following oxidative stress and necrosis or apoptosis in stained cells upon light exposure. PDT is currently used in oncoology. We studied the involvement of diverse inter- and intracellular signaling processes in PDT-induced death of crayfish neurons and surrounding glial cells (GC). Application of neurotrophic factors: NGF, BDNF, GDNF, CNTF, neurturin, neuregulin HRG1β, or EGF showed that only NGF and GDNF protected GC from PDT-induced apoptosis and necrosis. GDNF also protected neurons from PDT-induced necrosis. It preserved their mitochondrial ultrastructure and maintained the succinate dehydrogenase activity. This indicates the presence of receptors capable of recognizing mammalian NGF and GDNF in crayfish GC and neurons. Application of chemical NO generators and NO synthase inhibitors showed that NO, another intercellular mediator protects neurons from PDT-induced necrosis but increases PDT-induced apoptosis of glial cells. This effect was mediated by protein kinase G. Application of inhibitors or activators of a variety of intracellular signaling proteins demonstrated the involvement of calcmodulin, calcmodulin-dependent kinase II, adenylate cyclase and protein kinase B/Akt in PDT-induced necrosis of neurons and GC. Protein kinase C, MAP kinase p38 and tyrosine phosphatases were involved in PDT-induced necrosis of GC but not neurons. Tyrosine kinases participated in PDT-induced necrosis of neurons but not glia. On the other hand, protein kinases A and C were involved in protection of neurons from PDT-induced necrosis. In glial cells, phospholipase C mediated PDT-induced apoptosis of GC, whereas adenylate cyclase, protein kinases A and C and MAP kinase JNK, protected GC against PDT-induced apoptosis. Thus, various signaling pathways control not only apoptosis but also necrosis of glial cells and neurons. Necrosis is, therefore, not uncontrolled but the regulated cell death mode. Neurons and GC have different sets of signaling proteins involved in regulation of necrosis or apoptosis. The work was supported by grants of RFBR (11-04-01476) and Minobrnauki RF (4.6142.2011).

**SW04.S19–38**

Kidney-to-brain cross-talk as a way to ameliorate brain damage after brain ischemia

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Since certain brain pathologies can be attributed to the consequences of oxidative stress, antioxidant treatment may be an effective approach. Mitochondria-targeted antioxidants could rescue the ischemic organ by providing specific delivery of antioxidants molecules to the mitochondrion, which potentially suffers from oxidative stress more than other cellular compartments. We showed the neuroprotective effect of mitochondria-targeted antioxidant SkQR1 in a few models of brain ischemia. Also we demonstrate that SkQR1 is able to induce some elements of ischemic tolerance pathways such as an increase in erythropoietin (EPO) levels and phosphorylation of glycogen synthase kinase 3β (GSK-3β) in the kidney. The regulation of EPO production is believed to involve activation of the hypoxia-inducible transcription factor (HIF-1α), which depends on redox-sensitive stabilization of its α subunit. Redox-dependence of EPO expression allows speculating on existence of the mechanism of indirect neuroprotective action of antioxidants mediated by the kidney which is the main EPO-producing organ. To test this hypothesis we used ischemic preconditioning of the kidney that is believed to cause enhanced EPO release.

The goal of our study was to track the relations between the kidney and the brain in terms of the amplification of defense mechanisms during SkQR1 treatment and remote renal preconditioning and provide evidence that the kidney can generate signals inducing a tolerance to oxidative stress-associated brain pathologies.

We used a mitochondria-targeted antioxidant, SkQR1, as a potential agent to alleviate the deleterious consequences of stroke. Single injection of SkQR1 before cerebral ischemia in a dose-dependent manner reduced brain damage and improved functional recovery. Concomitantly, an increase in the levels of EPO in urine and phosphorylated GSK-3β in the brain was detected 24 hours after SkQR1 injection. However, protective effects of SkQR1 were not observed in rats with bilateral nephrectomy and in those treated with nephrotoxic antibiotic gentamicin, indicating the protective role of humoral factor(s) which are released from functional kidneys. Renal preconditioning also induced brain protection accompanied by an increased EPO level in urine and kidney tissue and P-GSK-3β in brain. Co-cultivation of SkQR1-treated kidney cells with cortical neurons resulted in enhanced phosphorylation of GSK-3β in neuronal cells.

The results indicate that preconditioning-induced and SkQR1-induced brain protection may be mediated through the release of EPO from the kidney.

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**SW04.S19–39**

Implication of ionotropic presynaptic glutamate receptors in the long-term sequelae of early life hypoxia and seizures

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Early life hypoxia and seizures can lead to development of epilepsy and other neurological deficits in adult life. Although neonatal seizures often do not immediately progress to chronic epilepsy, they do cause increased susceptibility to seizures and a risk of epilepsy later in life. Understanding of the molecular mechanisms involved in the effect of early life seizures on synaptic function, including epileptogenesis, will be critical in developing of appropriate therapies targeted on preventing of these long-term sequelae.

Here we report the data obtained by observations of rats exposed to hypoxia and seizures at the age of 10–12 postnatal days and taken in experiments 8–9 weeks after hypoxia treatment. Taking account of the several parameters the first two postnatal development weeks of the rodents brain are roughly equivalent to the neonatal period and early infancy in human brain development.

We are particularly interested in presynaptic modulatory systems that selectively modify the release of glutamate as well as inhibitory neurotransmitters such as GABA. We characterize the events induced by glutamate receptors agonists in isolated hippocampal nerve terminals by analyzing [3H]GABA release from...
nerve terminals in control and exposed to hypoxia/seizures rats. We have demonstrated that glutamate receptors located on hippocampal nerve terminals are implicated in a fine tuning of the neurosecretory process. In control animals, response to glutamate includes two processes: a rapid release of \(^{[3H]}\text{GABA}\) that is followed by its reuptake into nerve terminals. Using different agonists and antagonists of kainate and NMDA types of glutamate receptors we have provided evidence that activation of both receptors types stimulated \(^{[3H]}\text{GABA}\) release which is similarly to glutamate. After hypoxia/seizures exposure, \(^{[3H]}\text{GABA}\) release evoked by activation of presynaptic glutamate receptors is enhanced. The data allow to suggest that the main reason of this effect is attenuation of GABA transporter reuptake function. As a result, the activation of presynaptic glutamate receptors with natural agonist glutamate leads to increase of GABA outflow via the carrier-mediated mechanism. Kainate, but not NMDA, subtype of glutamate receptors are involved in this kind of modulation.

**SW04.S19-40**

**Neuronal tumor cells are sensitive to arginine amino acid deprivation**

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The effects of arginine deprivation – novel therapeutic approach to treat cancers, has been examined in more than 26 cell lines [1], but are no data what happens in neuronal tumors. Here we address the question of whether absence of arginine affects glioblastoma and neuroblastoma as well as primary culture rat glia cells.

Analysis of the effects of arginine deprivation have been shown profound differences between two lines. Glioblastoma U251MG cells have ability to survive until 144 hours in absence of arginine. But from the beginning of 48 hours we have observed evident changes in cell morphology, cell adhesion and cytoskeleton organization in comparison to cells grown on complete medium and a medium deprived of lysine (deprivation control cells). Moreover arginine deprived cells become less motile and invasive. Same effect we have shown in SHSY5Y neuroblastoma but already on 12 hours of deprivation. Analysis of cell cycle has been confirmed that neuroblastoma cells are more sensitive to arginine deprivation.

The observed changes were found in glioma but not in glia cells indicative of different arginine metabolism in transformed and physiological cells.

We believe that these changes in arginine-deprived cells are caused by deficit in actin arginylation as 2D-gel electrophoresis revealed lack of positively-charged \(\beta\)-actin isoforms.

To further test this we have examine level of polymerization of \(\gamma\)-actin-it is known non-arginylated [2]. And there is no differences in amount of filamentous \(\gamma\)-actin. In another hand it is dramatic reduction polymeric fraction of \(\beta\)-actin.

**References**


that is involved in several physio and pathological cell processes. In a healthy cell, GlcCer is a key lipid in the regulation of several processes. However, if there is an impairment in any of the machinery involved in GlcCer degradation (mainly in the lysosome), this lipid accumulates in the lysosomes and in other organelles. This is a hallmark of one of several Human metabolic diseases, the Gaucher disease (GD). Several studies have been made to explore the biology and biochemistry of this disease, but little attention has been given to the biophysical consequences of GlcCer accumulation, which likely contribute to lysosomal and other organelle malfunction that consequently can lead to the failure of the whole organelle and cell. It is therefore important to understand how GlcCer influence membrane biophysical properties. Our results show that GlcCer promotes strong alterations on the biophysical properties of model and cells membranes. In model membranes GlcCer promoted the formation of lipid domains displaying distinct (gel-like) phase properties and, in addition, induced strong morphological alterations. In cell membranes of fibroblasts from type I GD patients, there was a global decrease in membrane fluidity, consistent with the formation of GlcCer-enriched lipid domains and GlcCer-induced membrane packing. The changes observed were dependent on the type of mutation.

Our results highlight the impact of increased levels of GlcCer on membrane biophysical properties, further suggesting that alterations on membrane properties might be one of mechanisms underlying the pathophysiological changes in GD.

SW04.S19–43
Deciphering biochemical processes underlying cognitive functions and neuronal plasticity in the brain
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To elucidate the molecular mechanisms involved in formation of the cognitive functions during postnatal development of mammals we have analysed the effect of prenatal hypoxia (3 hours, 7% O2) and normal ageing on brain synaptic plasticity and memory in rats and on the activity of a neuropeptidase neprilysin (NEP) which represents a major amyloid-degrading enzyme in the brain. Previously we have demonstrated that hypoxia and ageing shift the balance of amyloid precursor protein (APP) and amyloid-β peptide (Aβ)-metabolising enzymes towards amyloidogenic pathways. We have also found that the average density of labile synaptotagmin-positive dendritic spines in the neocortex of rats exposed to prenatal hypoxia was decreased compared to controls. These rats also had decreased levels of NEP activity in the cortex and hippocampus. Similar decrease in the number of labile spines was observed in adult rats after i.c. injections of NEP inhibitors phosphoramidon and thiopran. A decrease in the number of synaptotagmin-positive spines was also observed in normally aged rats compared to younger individuals. Normal ageing was also accompanied by a decrease of NEP expression and activity in the cortex and hippocampus. In all cases (prenatal hypoxia, ageing and administration of NEP inhibitors) we have also observed deterioration of short- and long-term memory in rats. On the other hand, injections of valproic acid (an inhibitor of histone deacetylases) to adult rats, subjected to prenatal hypoxia, resulted in restoration of NEP activity, number of synaptotagmin-positive labile spines and improved short- and long-term memory. These data suggest that the activity of some enzymes (e.g. neuropeptidases) underlie neuronal network plasticity in the neocortex and their deficit can lead to functional cognitive disturbance (including dementia). Regulation of these enzymes via epigenetic mechanisms (e.g. histone deacetylase inhibitors) can be considered as a possible therapeutic strategy for improvement of cognitive functions impaired by prenatal stress or ageing. Supported by RFBR (grants 13-04-00388, 12-04-32281), Program of RAS ‘Fundamental Sciences to Medicine’, MRC UK, ARUK.

SW04.S19–44
Glycohydrolases and glycosphingolipids behavior in acid-sphingomyelinase knock-out mice
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Niemann-Pick type A (NPA) disease is a lysosomal storage disorder caused by a deficiency in the enzyme acid sphingomyelinase (ASMase). This inherited sphingolipidosis is characterized by a rapidly progressive neurodegenerative course.

It is now known that secondary to sphingomyelinase, other lipids, including gangliosides, also accumulate in NPA patients, leading to many cellular abnormalities. In particular we found that the lipid composition of different central nervous system and extraneural tissues from the acid sphingomyelinase-deficient mouse (ASMKO), the animal model for Niemann-Pick disease type A, is characterized by an unexpected, tissue specific selection of the accumulated molecular species of sphingomyelin, and an accumulation of GM3 and GM2 gangliosides in both neural and extraneural tissues, that cannot be solely explained by the lack of acid sphingomyelinase.

On these bases, we determine the glycohydrolytic enzyme panel in ASMKO mice as experimental model. The activity of different glycohydrolases was measured in the homogenates of brain, cerebellum, liver, spleen and testis from male mice at 1.5, 3 and 6 months. In particular, β-glucosidase GBA1, GBA2, β-galactosidase, β-hexosaminidase and Neu3 sialidase were evaluated. The data obtained revealed that the activity of most of the enzymes increased with the age in the majority of the organs analyzed in both wild type and ASMKO mice. The activity of β-glucosidase GBA1 and β-hexosaminidase activities were found to be significantly higher in ASMKO animals than in control mice; remarkably, such increase in the activity of both the enzymes was already detectable at the first age considered. In addition, an increase in the activity of β-galactosidase and Neu3 was also detectable in ASKMO mice at 6 months when compared to wild type animals of the same age.

Collectively, all these results demonstrated that the activity of several glycohydrolases is markedly altered in the organ of ASMKO mice, suggesting a possible role for some of these enzymes as earlier markers of NPA disease. In particular, GBA1 seems to represent a good candidate to be exploited for diagnostic and/or prognostic purposes. If validated with a larger number of animals, these data can provide a new tool for a better understanding of the etiology of NPA disease.
SW04.S19–45
Development of a novel Parkinson’s disease model based on methylotrophic yeast Hansenula polymorpha
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Accordingly to data of the World Health Organization there are 4 million patients with Parkinson’s disease (PD) in the world. Among the disorders of the central nervous system, Parkinson’s disease has second position after Alzheimer’s disease. It is known that these disorders have common cellular and molecular mechanisms including protein aggregation and inclusion body formation. Abnormal accumulation of synuclein in neuronal cell bodies, axons, and synapses is the main event in PD.

Intense research has been conducted to generate artificial models for investigation of PD and development of new therapeutic approaches for this pathology because of study of molecular mechanisms of human synucleinopathy and methods of their correction are painful for realization in vivo. Several features of neurodegenerative diseases, such as formation of protein aggregates, cellular toxicity mediated by misfolded proteins, oxidative stress and hallmarks of apoptosis have been faithfully recapitulated in yeast, enabling researchers to take advantage of this powerful model to rapidly perform genetic and compound screens with the aim of identifying novel candidate therapeutic targets and drugs. Evidence from prospective epidemiological studies has identified diabetes mellitus as an independent risk factor for multiple diseases of the nervous system. It has been reported that around 50–80% of PD patients have abnormal glucose tolerance and that diabetes is associated with an increase of PD risk.

We are creating novel yeast models of PD with regulated SNCA gene expression. These models are based on H. polymorpha wild type strain and mutants defective in glucose transport and metabolism. Combination of defective glucose metabolism and α-synuclein overproduction in single strain will help us to understand relationship between Parkinson’s disease and abnormal sugar metabolism. We will induce and regulate the autophagic process for α-synuclein degradation in isolated H. polymorpha strains by limitation of nitrogen source. We hope that autophagic degradation will positively result in vacuole-mediated amyloid disruption and in future it could be applied as a possible new strategy for novel therapeutic approaches.

SW04.S19–46
Interdependence of amyloid formation in yeast: significance for amyloid pathology
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Amyloids are highly stable and insoluble fibrillar protein aggregates with a specific cross-beta structure, which often cause incurable diseases in humans and animals. An important class of such diseases involve amyloid aggregation of proteins with elongated polyglutamine tracts (polyQ). Notably, longer polyQ are associated with more rapid amyloid formation and faster disease progression. One of the most common polyQ disorders, Huntington disease, is caused by polyQ expansion in the huntingtin protein. Using a well-characterized yeast model of Huntington disease, we show that the toxicity of human huntingtin with expanded polyQ (103Q) is substantially caused by depletion of essential translation termination factors Sup35 (eRF3) and Sup45 (eRF1). This depletion results from the amyloid polymerization of Sup35 seeded by huntingtin amyloids and related sequestration of Sup45, which interacts with Sup35 polymers. Surprisingly, huntingtin with non-pathogenic polyQ (25Q) can also polymerize when seeded by amyloids of other Q-rich proteins and form toxic amyloids, thus indicating that under certain circumstances diseases can be caused by proteins with non-expanded polyQ.

To reveal additional sources of huntingtin toxicity, we elucidated which proteins, besides Sup35, are converted into polymeric form in the presence of huntingtin amyloids. For this, we isolated SDS-insoluble protein aggregates from yeast cells containing huntingtin amyloids. The isolated proteins were identified using 2D-electrophoresis and subsequent mass-spectrometry. Nearly all of these proteins had Q-rich domains, and some of them have been previously shown to be implicated in huntingtin toxicity in yeast. This shows that the used method can reveal the repertoire of proteins whose polymerization is induced by pathogenic amyloids and, in perspective, help determine the role of amyloid cross-seeding in human amyloidoses.

SW04.S19–47
Small-angle X-ray scattering studies of E3 ligase parkin
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Parkinson’s disease (PD) is the most common neurodegenerative movement disorder characterized by loss of nigral dopaminergic neurons. The mutations of the Parkin gene (PARK2), leading to the accumulation of misfolded proteins in neurons have been unambiguously account for the majority of autosomal recessive juvenile PD (ARJP) and or more than 15% of sporadic PD cases with early onset [1]. The mammalian PARK2 gene encodes 425-amino acids of 52-kDa parkin protein comprising six domains. Parkin functions in vitro and in vivo as an E2-enzyme dependent RING ubiquitin E3 ligase [2] – a component of the ubiquitin-protein- and the ubiquitin-proteasome system (UPS). The parkin loss-function mutations of E3 ligase activity has been recognized to play a central role in molecular basis of PD pathogenesis. The alternative splicing variants of the parkin gene might display different functions and regional distribution and could be also associated to the ARJP phenotype [3].

Despite the intense interest in understanding parkin function, so far no data are available about it spatial organisation. The present small-angles X-ray scattering studies have been employed to provide for the first time the information on the overall shape of full-length parkin and recombinant protein comprising parkin transcript variant TV7.

PCR products of synthesized parkin gene (GeneArt) and parkin comprising TV7 splice variant were cloned into bacterial vectors pGEX-6P1, pGEX-4T1. Proteins were overexpressed in E. coli strains, isolated and purified using affinity GST-chromatography followed by size exclusion chromatography. The small angles X-ray scattering (SAXS) data were collected at...
DESY (Hamburg, Germany) and ESRF (Grenoble, France) synchrotron sources. The SAXS data was collected from full-length GST fusion parkin, full-length parkin without fusion tag (GST) cleaved with suitable protease and from recombinant fusion GST protein containing TV7 parkin splice variant. Data processing and analysis was done using programs of the ATSAS suite (http://www.embblhamburg.de/biosaxs/). The ab initio small angles scattering models structures of all sample proteins were calculated using Dammin program [4]. The shape of constructed models allowed to suggest the mutual positions of the parkin ubiquitin-like domain structure (ZEEQ), two homologue RING domains structures (2VJE) and parkin IBR structure solved by NMR (2JMO).

References

SW04.S19–48
Overexpression of DYRK1A inhibits choline acetyltransferase induction by oleic acid in cellular models of Down syndrome
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Histological brain studies of individuals with DS have revealed an aberrant formation of the cerebral cortex. Previous work from our laboratory has shown that oleic acid acts as a neurotrophic factor and induces neuronal differentiation. In order to characterize the effects of oleic acid in a cellular model of DS, immortalized cell lines derived from the cortex of trisomy Ts16 (CTb) and normal mice (CNh) were incubated in the absence or presence of oleic acid. Oleic acid increased choline acetyltransferase expression (ChAT), a marker of cholinergic differentiation in trisomic cell lines similar to those of normal cells in the presence of oleic acid. In agreement with these results, oleic acid was unable to increase ChAT expression in neuronal cultures of transgenic mice overexpressing DYRK1A. In summary, our results highlight the role played by DYRK1A in brain development through the control of ChAT expression. In addition, the overexpression of DYRK1A in DS models prevented the neurotrophic effect of oleic acid, a fact that may account for mental retardation in DS patients.

SW04.S19–49
Evidence for the implication of L-Dopa decarboxylase in apoptosis
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L-Dopa decarboxylase (DDC) is the enzyme that catalyzes the decarboxylation of L-Dopa to dopamine. One of the earliest detectable events during apoptosis is the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane and the binding of Annexin V to PS with high affinity. For this reason, Annexin V is considered as an apoptotic marker. Studies from our laboratory have indicated that Annexin V and DDC interact in human cells of neural and non-neural origin. Based on this observation, in the present study we investigated the effect of DDC expression in apoptosis. CHO (Chinese Hamster Ovary) cells expressing different human DDC isoforms, were subjected to Staurosporine (STS)-induced apoptosis. Our results indicated a significant increase in cellular death in CHO cells expressing the full length DDC molecule following STS treatment, as compared to controls. The same phenomenon was observed for CHO cells expressing truncated human DDC isoforms. In order to further investigate the possible involvement of DDC in apoptosis, we proceeded to study whether DDC interacts with other molecules that are involved in apoptotic mechanisms. It is well established that the Bcl-2 family of proteins regulate apoptotic cell death. Our immunoprecipitation experiments demonstrated a clear interaction of DDC with Bcl-2, which was reduced under apoptotic conditions in human cells. Furthermore, the interaction between DDC and Annexin V was altered under apoptotic conditions. The above data strongly suggest an implication of L-Dopa decarboxylase in apoptosis through a yet unknown mechanism. The further investigation of the above observations is important in order to study the pathogenesis of conditions in which DDC is involved and are characterized by impairment of apoptotic mechanisms such as cancer and neurodegeneration.

SW04.S19–50
Polychlorinated biphenyls decrease glial fibrillary acidic protein expression during dibutyryl cAMP-induced astrocytic differentiation
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Polychlorinated biphenyls (PCBs) are persistent and widespread environmental pollutants that exert neurotoxic properties [1]. In this study, we investigated the effects of a commercial mixture of PCBs, Aroclor1254 (A1254), on glial neurotoxicity and astrocytic differentiation using the rat C6 glial cell line. To determine PCBs cytotoxic effects, C6 cells were exposed to medium containing different concentrations of A1254 (0.05–90 μM), 0.1% dimethyl
It is important to determine reliable serum bio-


significantly lower than that of healthy age matched controls

effort is needed to conduct large scale studies in different popu-


determined with ELISA.

controls (age

recruited according to the DSM-IV criteria. Age matched healthy

suggested to participate in almost all major neurodegenerative

generative and inflammatory brain disorder. Complement activa-

that CP may produce neurosteroids as well, but a recent cDNA

tissue. Unlike other brain regions, there are no current indications

that CP is a target of sex steroid hormones, which regulate the

roles in repair processes following trauma. There are evidences

pounds from the peripheral blood into the CSF, and play pivotal

in protecting the brain from insults such as neurodegeneration or

ischemia. The choroid plexus (CP) forming a physical interface

between the peripheral blood and the cerebrospinal fluid (CSF),

regulate the uptake of nutrients, hormones and several other com-

pounds from the peripheral blood into the CSF, and play pivotal

roles in repair processes following trauma. There are evidences

that CP is a target of sex steroid hormones, which regulate the

expression of some proteins with impact in neuroprotection in this

tissue. Unlike other brain regions, there are no current indications

that CP may produce neurosteroids as well, but a recent cDNA

microarray analysis of the CP transcriptome showed that several

enzymes involved in steroidogenesis are expressed in this tissue.

Thus, here, we investigate whether the key steroidogenic enzymes

P450scc, P450aro, 17βHSD3, 17βHSD8, 5α-reductase 1 and 5α-

reductase 2 are present in the CP and capable of generating neu-

rosteroiids. Consistent with our hypothesis, the present study dem-

onstrates mRNA and protein expression of these enzymes in rat

CP. Moreover, we also demonstrate that CP explants are capable

of the proton gradient of synaptic vesicles by the pro-

the level of membrane cholesterol attenuated transporter-

mented glutamate release from nerve terminals. Thus, here, we

investigate whether the key steroidogenic enzymes

that CP may produce neurosteroids as well, but a recent cDNA

tissue. Unlike other brain regions, there are no current indications

that CP is a target of sex steroid hormones, which regulate the

expression of some proteins with impact in neuroprotection in this

tissue. Unlike other brain regions, there are no current indications

that CP may produce neurosteroids as well, but a recent cDNA

microarray analysis of the CP transcriptome showed that several

enzymes involved in steroidogenesis are expressed in this tissue.

Thus, here, we investigate whether the key steroidogenic enzymes

P450scc, P450aro, 17βHSD3, 17βHSD8, 5α-reductase 1 and 5α-

reductase 2 are present in the CP and capable of generating neu-

rosteroiids. Consistent with our hypothesis, the present study dem-

onstrates mRNA and protein expression of these enzymes in rat

CP. Moreover, we also demonstrate that CP explants are capable
of converting \[^{1}\text{H}]\text{-androstenedione}\) to testosterone, using thin layer liquid chromatography, via 17|\text{HSD} enzyme. Collectively these data show that the CP has the potential to synthesize neurosteroids, that may be released in to the CSF and contribute to brain neuroprotection.

**SW04.S19-54**  
Erythrocyte SOD1 activity-role in bioavailability of NO and possible cause of 'duing back' phenomena in neurodegenerative diseases  
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The auto-oxidation of oxyhaemoglobin to methaemoglobin generating superoxide anion radical (O\(_2^-\)) represents the main source of free radicals in the erythrocytes. However human erythrocytes are also exposed to nitric oxide (NO) from circulation. Since there is no de novo synthesis of SOD1 enzyme in mature erythrocytes it induces reduction defence capacity for O\(_2^-\) dismutation and increase elimination of NO from circulation through the reaction between O\(_2^-\) and NO which than occurred in cells producing peroxynitrite (ONOO\(^-\)), a cytotoxic molecule. When erythocyte SOD1 decreased the consequence should be increased ONOO\(^-\) and decreased NO bioavailability for preventing muscle hypoxia. We measured SOD1 activities in erythrocytes from different patients characterized with muscle deterioration such as amyotrophic lateral sclerosis (ALS) and myotonic dystrophy type 1 (MD1). In all examined groups we found significantly decreased SOD1 activity in comparison with controls. Even more SOD1 from these samples showed decreased stability toward hydrogen peroxide (H\(_2\)O\(_2\)) induced inhibition as well as increased free radical production in the presence of H\(_2\)O\(_2\). Although there is different origin of SOD1 decreased activity (mutation or metabolic inhibition) there are similar consequence -muscule weakness and atrophy. Fundamental new metal – and nitrite-catalyzed chemical reaction pathways that generate free NO, N\(_2\)O\(_3\) and nitroso thiol which constitute the basis of in vivo nitrite-dependent hypoxic signal transduction could be operative only under conditions with optimal SOD1 activity. The measurements of changes in the activities of SOD1 is capable of providing a valuable insight into the complexity of overall relations leading to the decreased NO bioavailability and its relevance to the systemic effects.

**SW04.S19-55**  
Iron activates 5-lipoxygenase and induces its nuclear translocation: implications for the pathogenesis of Alzheimer  
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The etiology of Alzheimer’s disease (AD) progression is still debated, however, increased oxidative stress is a sustained event that underlies much of the neurotoxicity and consequent neuronal loss. AD is also associated with an abnormal accumulation of some metal ions, and recently it has been shown that one of these, iron, plays a relevant role in affecting neurotoxicity. 5-Lipoxygenase (5-LOX) is an iron-containing dioxygenase that catalyzes the two initial steps in the biosynthesis of leukotrienes (LTs), a group of inflammatory lipid mediators. To ascertain whether iron can modulate trafficking and activity of human 5-LOX, we exposed THP-1 macrophages to exogenous ferric iron (Fe\(^{3+}\)) or hemin, which is rapidly degraded through the catalytic activity of heme oxygenase leading to the release of free iron. Furthermore we investigated the expression and activity of 5-LOX in peripheral blood mononuclear cells (PBMCs) of subjects with late-onset AD (LOAD) and age-matched controls. Cells exposed to increasing amounts (from 0.1 to 100 \(\mu\)M) of Fe\(^{3+}\) for 5 min, showed a dose-dependent increase of 5-LOX activity, in parallel with the redistribution of the cytosolic 5-LOX to the nuclear fraction, assessed by means of subcellular fractionation and by confocal microscopy analysis. In addition, priming cells with exogenous hemin recapitulated the effects of free iron on 5-LOX translocation and activity. Treatment with the iron-chelating agent deferoxamine, completely abolished the hemin-dependent translocation of 5-LOX to the nuclear fraction.

We found a significant increase in 5-LOX gene expression in AD subjects compared to healthy controls, that was paralleled by increased 5-LOX protein and leukotriene B\(_4\) (the product of 5-LOX enzymatic activity). In addition, a consistent reduction in DNA methylation at 5-LOX gene (Alox5) promoter was documented in AD versus healthy subjects, and a significant correlation between 5-LOX expression and circulating levels of heme was found.

These results suggest that iron modulates 5-LOX activity, by increasing the capacity of the enzyme to bind nuclear membranes. Our data also support the hypothesis that 5-LOX overexpression might contribute to increase brain vulnerability to neurodegeneration where iron homeostasis is altered.

**SW04.S19-56**  
Novel 2-pyrazoline derivatives bearing thiazole ring as dual monoamine oxidase-B and acetylcholinesterase inhibitors also inhibit beta-amyloid fibril  
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Alzheimer’s Disease (AD) is a neurodegenerative disorder that is the major cause of dementia amongst the elderly. Since the loss of cholinergic neurons are distinctive in the brains of AD patients, cholinesterase inhibitors are used to increase cholinergic transmission and improve cognitive performance in these patients. Selective monoamine oxidase-B (MAO-B) inhibitors have been shown to protect neuronal cells from oxidative stress and slow the progression of the disease in AD patients.

In the present study, seven novel pyrazoline derivatives bearing thiazole ring which have been previously synthesized and determined as selective and potent MAO-B inhibitors by our group were tested for their potential to inhibit human acetylcholinesterase (AChE). Preliminary inhibition studies showed that all compounds tested inhibited AChE with IC50 values varying between 12 and 24 \(\mu\)M. These Compounds also inhibited beta amyloid fibril formation from the Ab(1-40) and Ab(1-42) peptides in vitro. Although the mechanism of their interaction with beta amyloid fibrils are not fully deciphered yet, these new MAO-B
and AChE dual inhibitors appear as the promising molecules for the development of potential therapeutics for AD.

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SW04.S19–57
DNA-abzymes selected from the phage display library of antibodies of multiple sclerosis patients
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Blood sera of patients with autoimmune diseases have been shown previously to possess catalytic antibodies of various specificity, participating in the pathogenesis of immune disorder. One possible way for isolating human monoclonal antibodies of this kind is selecting them from phage display libraries of antibodies (single chain variable fragments (scFv) or Fab-fragments). Autoimmune library of scFv from peripheral blood lymphocytes of multiple sclerosis (MS) patients was analysed for the presence of DNA-abzymes. Library was panned against heparin-agarose and derived populations were screened for ability to hydrolyze supercoiled plasmid. Two phage antibodies were selected and converted to fully human recombinant IgG1 (fh IgG1). The purified preparations of both fh IgG1were tested for ability to interact with supercoiled plasmid. The effect observed indicates the ability of both fh IgG1 to hydrolyze dsDNA. Probably, catalytic antibodies of this kind participate in MS pathogenesis.

SW04.S19–58
Effect of genotype structure for 5 MYOC gene SNPs on its frequency in patients with adult-onset Primary Open Angle Glaucoma (POAG)
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Primary open angle glaucoma (POAG) is a chronic neurodegenerative eye disease caused by degeneration of axons of the retinal ganglion cells (RGC) and subsequent neurons death. The disease can occur at any age, but individuals after 40 are most susceptible to it. The etiology of POAG remains unknown. The aim of our study is to develop an algorithm of the search for candidate genes that can potentially be involved in the development of pathological phenotype of POAG in individuals after the age of 40. The analysis of existing experimental data in the literature demonstrates that multifactorial effect on retinal ganglion cells during all optical pathway can not be excluded. A possibility of the effect of particular violations in different intra- and intercellular molecular interactions, changes in macromolecules and mitochondria transport in RGC axons, state of blood-brain barrier and axon repair mechanisms, apoptosis, level of neurototoxicity, etc., on the origin of pathophysiological process is considering.

Based on the suggested approach, we are sequentially selecting the candidate genes. We selected the MYOC gene as a candidate gene; myocilin protein, which refers to olfactomedin domain-containing family of proteins (that play an important role in neurogenesis) is the product of this gene. The MYOC gene expression was found almost in all tissues of the visual organ; its presence in mitochondria was also demonstrated. In the present study, the preliminary data on five MYOC gene single nucleotide polymorphisms (SNPs) are presented (~1000C/G, ~83G/A, G227A (Arg76Lys), IVS2(+35G/A), T1041C (Tyr347Tyr)). We studied 149 patients with adult-onset POAG (age after 40); 187 healthy for this disease individuals (after 40) were used as a control group. Two studied SNPs (~83G/A and G227A (Arg76Lys)) are in linkage disequilibrium. As a result of our analysis, a significant decrease in the frequency of the GG/GG(GG)/AA/TC genotype in the group of patients compared to the control group was detected (OR = 0.195, 95% CI 0.043–0.887, p = 0.026). The data obtained apparently indicate the heterogeneity of patient sample for differences in factors that affect the occurrence of adult-onset POAG.

SW04.S19–59
The effects of intracerebroventricular administration of STZ on insulin signaling pathway in rat brain
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The neurodegeneration that occurs in sporadic Alzheimer’s disease (SAD) is consistently associated with a number of characteristic histopathological, molecular and biochemical abnormalities, including cell loss, amyloid-β deposits, increased activation signaling pathways and impaired energy metabolism/mitochondrial function and the brain. This latter enzyme regulates glycogen synthase kinase (GSK)-3 which involve the production of amyloid-β peptides and phosphorylation of tau. In this study we aimed to investigate that the effects of insulin signaling mechanism on tau phosphorylation after intracerebroventricular (i.c.v) streptozotocin (stz) treatment in rat brain tissue. Wistar rats were assigned randomly into three groups, Group I: Control, Group II: SAD (i.c.v with stz 1 mg/kg), Group III: SAD+Insulin (i.c.v with stz 1 mg/kg+2 IU insulin s.c). Serum insulin and Amyloid β 1-42 (Aβ) levels were measured by ELISA. Using Western blot there were shown alterations of the insulin receptor (IR) signaling cascade at the level of PKB/Akt, GSK-3 and tau protein in the hippocampus, 1 months after i.c.v injection of stz in rats. Cognitive functions were tested by using a Morris water maze swimming test. Our results pointed out an increased Aβ levels, tau hyperphosphorylation and impairment in cognitive function, suggested that the defects of insulin signal transduction in brain could trigger or be a mediator of SAD.

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SW04.S19–60
A longitudinal microscope-based methodology to assess the effect of alpha-synuclein pathological mutations on stability and survival in primary cortical neurons
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Abnormal accumulation of alpha-synuclein (a-syn) into Lewy Bodies is a key neuropathological feature of a heterogeneous group of disorders (synucleinopathies) including Parkinson disease (PD) and dementia with Lewy Bodies (DLB) among others. Although 90% of PD cases are sporadic and linked to mutations on the gene encoding a-syn (SNCA) cause autosomal dominant PD. Indeed, an abnormal increase in a-syn levels is sufficient to cause Parkinsonism with prominent dementia, as observed in familial cases with genomic duplications and triplications of SNCA gene. Therefore, a-syn levels constitute a predictive factor of neuronal death. Yet, the mechanisms that determine a-syn steady-state levels on sporadic and familial PD cases with point mutations on the SNCA coding region remain unclear. It has been hypothesized that point mutations and/or posttranslational modifications on a-syn alter the stability of the protein leading to an increase of its steady state levels and eventually neuronal death. To test this hypothesis we used a microscope-based methodology to longitudinally track individual neurons and determine the risk of neuronal death induced by wild-type (wt) and mutant versions of a-syn. In order to determine the stability of wt and mutant versions of a-syn in living neurons we applied a novel optical pulse-chase methodology based on the photoswitchable protein Dendra2 and longitudinal analysis to measure its half-life on neurons in situ. Among the pathological a-syn mutations, the E46K mutation increases significantly the risk of neuronal death on primary cortical neurons. We are currently analyzing whether this effect is associated with a change on the stability of the protein.

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SW04.S19–61
Protective effect of GD1a and GM1 gangliosides against the toxic action of bacterial lipopolysaccharide on neuronal and epithelial cells
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Gangliosides are sialic acid-containing glycosphingolipids being especially abundant in the nerve cell membrane. They are localized primarily in lipid rafts, subdomains of the plasma membrane involved in the regulation of signal transduction. In different models of neurotoxicity, gangliosides were shown to possess neuroprotective properties. The present work was focused on the study of the influence of GD1a and GM1 gangliosides on toxic effect of bacterial LPS on two cell types: PC12 neuronal cell line and primary cultured epithelial cells from the frog urinary bladder (FUBEC). We found that both PC12 and FUBEC express TLNR4, a receptor of LPS. Treatment of PC12 cells incubated in serum-free medium with LPS (0.125 or 0.25 mg/ml) for 24 hours strongly decreased cell viability. Gangliosides were found to have a protective effect. Thus, 100 μM GD1a or GM1 increased the viability of PC12 exposed to 0.25 mg/ml LPS from 1.4 ± 0.9% to 64.6 ± 3.7% and 65.4 ± 11.5%, respectively. Also, preincubation of PC12 with gangliosides eliminated effect of LPS on ROS accumulation and iNOS expression. In FUBEC, LPS (25 μg/ml) slightly reduced cell viability, however, it strongly enhanced iNOS protein expression and increased NO synthesis. Pretreatment of FUBEC with GD1a or GM1 inhibited both iNOS expression and NO production. To clarify steps of LPS signaling targeted by gangliosides, we subjected PC12 and FUBEC to sucrose gradient centrifugation and analyzed TLNR4 expression in fractions corresponded to lipid rafts which were identified by flow-in and cholesterol enrichment. LPS was found to induce recruitment of TLNR4 into the lipid rafts. GM1 and GD1a prevented this effect of LPS suggesting the influence of gangliosides on lipid raft structure. This suggestion is supported by the facts that (i) exposure of the cells to GM1 and GD1a led to their incorporation into the cell membrane; (ii) methyl-f-cyclodextrin, a raft-disrupting agent, also abolished LPS-triggered TLNR4 translocation to the lipid raft in both cell types. Taken together, these data indicate that gangliosides protect cells against the toxicity by preventing translocation of TLNR4 into the lipid rafts and subsequent activation of downstream signaling molecules.

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SW04.S19–62
The swiss cheese mutants develop morphological and functional changes in neuromuscular junctions of Drosophila melanogaster
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Hereditary spastic paraplegia (HSP) is a heterogenic group of the diseases leading to the development of the lower limb paralysis. NTE human gene is involved in the development of the autosomal recessive form of the hereditary spastic paraplegia (HSP SPG39). NTE human gene is also a molecular target for organophosphorus compound poisoning leading to the development of organophosphorus induced delayed neuropathy (OPIDN).

Both HSP and OPIDN are characterized by the distal neurodegeneration of motor and sensitive axons. Pathological mechanisms underlying the HSP and OPIDN development are poorly understood today despite the amount of research carried out on the subject. One of the main experimental solutions of studying the functions of human genes is to study their orthologs in model animals. Swiss cheese (sws) is an ortholog of human NTE in Drosophila melanogaster. Sws mutants are characterized by progressive neurodegeneration and glia disruption in a brain and early death of the adult animals.

To study sws functions we chose neuromuscular junctions (NMJs) of the 3rd instar larvae of Drosophila melanogaster, the system allowing analysis of changes in a single neuron. In this study we examined the sws mutants: sws0, sws7615, sws2612. Using methods of genetics, immunohistochemistry and confocal microscopy we showed that sws plays an important role in processes of NMJs formation and functioning. Mutations in sws alter...
the size and the number of big synaptic boutons and the number of the satellite boutons and cause abnormal distribution of synaptic proteins in NMJs of Drosophila melanogaster. Axonal transport analysis showed a severe alteration of mitochondrial clusters and synaptotagmin quantity in axons of sws mutant larvae.

SW04.S19–63
The role of the prion protein in neurodegenerative disorders

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The cellular prion protein (PrP<sup>C</sup>) is a cell surface glycoprotein mainly expressed in the central nervous system. A β sheet-reach conformer of PrP<sup>C</sup> generates the prion, the infectious protein causing prion diseases. The physiological role of PrP<sup>C</sup> remains enigmatic although it has been suggested that PrP<sup>C</sup> participates in Ca<sup>2+</sup> movements in the plasma membrane, cytosol and the mitochondrial matrix of cerebellar granule neurons (CGN) derived from PrP-KO (PrP-KO) mice and, as controls, from Tg46 mice in which normal PrP<sup>C</sup> levels were rescued over a PrP-KO genotype mice. We found that, compared to controls, PrP-KO neurons have significant increased Ca<sup>2+</sup> transients in all the above domains after activating both store-operated Ca<sup>2+</sup> channels (SOCC), glutamate and/or NMDA receptors (R) and that Aβ affects neuronal local Ca<sup>2+</sup> fluxes in a PrP<sup>C</sup>-dependent way. Because of the involvement of p59<sup>α</sup> and p42/p44 ERK (ERK 1/2) in regulating SOCC and the NMDA-R (Pozo-Guisado et al., 2010, Nakazawa et al., 2001), we then explored the possibility that PrP<sup>C</sup> modulates SOCC and the NMDA-R through p59<sup>α</sup> and ERK 1/2 signaling pathways. We found that PrP-KO CGN had higher levels of active p59<sup>α</sup> before activating both SOCC and NMDA-R and increased phosphorylated ERK 1/2 only under the conditions that preceded NMDA-R activation. The difference of phosphorylated p59<sup>α</sup> observed in untreated neurons before stimulating SOCC was abrogated by the presence of Aβ (1-42) fragments. These preliminary results indicate that p59<sup>α</sup> and ERK 1/2 participate in the PrP<sup>C</sup>-dependent modulation of Ca<sup>2+</sup> movements via SOCC and NMDA-R, and that PrP<sup>C</sup>-Aβ interactions increase SOCC-mediated Ca<sup>2+</sup> transients by abolishing the PrP<sup>C</sup>-dependent downregulation of the p59<sup>α</sup> pathway.

SW04.S19–64
Intersectin adaptor proteins and pathologies

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Intersectins (ITSN) is a family of endocytic adaptor proteins that have a considerable impact in functioning of a nervous system and are associated with neurodegenerative diseases and cancer. Changes of ITSN1 expression level or disruption of its function were reported to affect synaptic vesicle endocytosis and dendritic spine morphology. Overexpression of ITSN1 gene is associated with development of Down syndrome and Alzheimer’s disease. ITSN2 has been proposed to be a predictive marker for breast cancer. High expression level of ITSN2 was associated with prolonged disease-free survival in breast cancer patients after adjuvant chemotherapy with cyclophosphamide, methotrexate and 5-fluorouracil.

We analyzed expression of short and long IHSN2 isoforms in breast tumors. Lowest level of IHSN2 expression, especially IHSN2-S isoform, was detected in estrogen/progesterone receptor-negative, HER2/neu-positive tumors with poor prognosis and in estrogen/progesterone receptor-positive tumors of patients with lymph node metastasis. Amount of IHSN2-L did not differ significantly in breast tumors analyzed.

In order to determine the composition of neuronal IHSN1-related protein complexes we performed search for novel IHSN1 neuron-specific interacting proteins. For SH3A domain we observed an unknown band of 125 kDa which was identified by MALDI-TOF mass spectrometry as STOP (stable tubule-only polypeptide). Since glial cells contain STOP isoforms with smaller molecular weight, we concluded that STOP is neuron-specific binding partner of IHSN1. The results of mass spectrometry were confirmed by co-immunoprecipitation of IHSN1-SH3 complex from mouse brain lysate. Finally, with both fluorescent antibodies and recombinant FP-tagged proteins we determined subcellular localization of these proteins in rat primary hippocampal neurons and found that they are partially co-localized in soma and dendrites.

We also investigated new ways of regulation of IHSN1 functioning. Since IHSN1 is a synaptic protein, we considered that it can possibly undergo Ca-dependent post-translational modifications. This suggestion was supported by bioinformatic predictions. To test this hypothesis, we performed in vitro kinase reaction between purified SH3 domains and coiled-coil region of IHSN1 and fraction of calmodulin-binding proteins from mouse brain lysate. In the presence of Ca and calmodulin both proteins were phosphorylated. To find the phosphorylation sites, we performed tandem mass spectrometry analysis of phosphorylated proteins and identified nine sites of serine/threonine phosphorylations. This suggestion was supported by bioinformatic predictions. To test this hypothesis, we performed in vitro kinase reaction between purified SH3 domains and coiled-coil region of IHSN1 and fraction of calmodulin-binding proteins from mouse brain lysate. In the presence of Ca and calmodulin both proteins were phosphorylated. To find the phosphorylation sites, we performed tandem mass spectrometry analysis of phosphorylated proteins and identified nine sites of serine/threonine phosphorylations, five for coiled-coil region and four for SH3 domains.

This work was partially supported by Joint Project of NAS of Ukraine and Russian Foundation for Basic Research (16-04-12U), Target complex interdisciplinary programme of scientific researches of NAS of Ukraine ‘Fundamentals of molecular and cell biotechnologies’ and State Fund for Fundamental Researches (Project F33.4/001).

SW04.S19–65
11S regulator has an influence on degradation of polyQ containing protein by proteasome

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Proteasome functions in every eukaryotic cell, degrading most of the irregular or nonviable cellular proteins. Turning off proteasome leads to disastrous consequences, and finally to cell death. The polyglutamine (polyQ) repeat diseases are inherited neurodegenerative disorders attributed to elongation of polyQ fragments (from 6–35 to 36–306 glutamine residues) in certain proteins. These abnormal proteins form aggregates and become insoluble, resulting in cytoplasmic or nuclear protein inclusions in some...
brain regions. Analysis of inclusions reveals presence of mutant proteins, and also of ubiquitin and components of 26S proteasome (20S core, 19S and 11S regulatory particles). It is assumed that proteasomal degradation system attempts to digest abnormal misfolded proteins, but fails to cut within polyQ regions or does it with low efficiency. Then proteasome may become clogged and inactivated by these long aggregated polyQ sequences. The aim of our study was to investigate the effect of the 11S regulator on proteasomal degradation of polyQ-containing protein, namely huntingtin (htt). 20S, 26S proteasome and 11S regulator were isolated from mouse brains. Homogenates were subjected to stepwise salt fractionation with subsequent purification by different chromatographic methods (gel filtration and anion exchange chromatography). Isolated proteins were characterized by molecular weight using gel-filtration and Western-blot. The dependency of 20S proteasome peptide activity on concentration of 11S regulator was examined. Kinetic constants of proteasome hydrolysis of three peptide substrates (Suc-L-LYY-AMC, Z-LLE-AMC and Ac-RLR-AMC) were studied in presence of 11S regulator. HEK cells were transfected by vector containing 15Q or 138Q htt gene (generous gift of Prof. E.V. Kaznacheeva, St.-Petersburgh) and cell lysates were subjected to hydrolysis by 20S proteasome with or without 11S regulator. Reactions were followed by Western-blot (anti-htt antibodies were kindly provided by Vladimir A. Vignat). Our data suggest a possible acceleration of proteasomal degradation of htt in the presence of the 11S regulator.

**SW04.S19–66**

**Modeling of the early clinical stage of Parkinson’s disease for testing of anti-parkinsonian drugs**

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Parkinson’s disease (PD) is characterized by a progressive degeneration of nigrostriatal dopaminergic (DA-ergic) neurons with no motor dysfunction for a long time because of the brain plasticity. An appearance of motor symptoms manifests irreversible degradation of the nigrostriatal system and a depletion of compensatory processes. This study was aimed to model the PD early clinical stage for testing of new anti-parkinsonian drugs. The early clinical stage of PD was modeled in mice by four-fold systemic injections with 2 hours intervals of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (12 mg/kg), which is converted to 1-methyl-4-phenylpyridinium (MPP+, toxin) in the brain. The number of the nigral tyrosine hydroxylase (TH)-immunoreactive (DA-ergic) cell bodies was decreased from the 3rd h after the last injection, reaching threshold axon degradation (70%) 3 hours later and remaining at this level for 2 weeks, the whole studied period. Axonal loss began earlier and lasted up to the 24th h following the last injection, reaching threshold axon degradation (70%). Survived neurons were characterized by: (i) the increased number of striatal axonal terminals, apparently, because of the axon ramification; (ii) the increased amount of dopamine (DA), TH and enhanced TH activity in individual cell bodies; (iii) the decreased amount of DA and TH and increased TH activity in the axons. Some data suggest a compensatory increase of the neuron functional activity (i), (ii) while others (iii) manifest an impairment of the axonal transport resulting in a decrease of DA synthesis. Taking into account that MPP+ is captured by the DA membrane transporter, the specificity and suitability of our model for testing of anti-parkinsonian drugs was checked with nomifensine, an inhibitor of DA uptake. Indeed, nomifensine was shown to prevent to a high degree the MPP+ toxic action on DA-ergic neurons. Thus, we have developed an experimental model of the PD early clinical stage, which is suitable for testing of anti-parkinsonian drugs.

**SW04.S19–67**

**Modeling of the inducer-dependent aggregation of tau protein**

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Taufopathies are neurodegenerative disorders encompassing various pathologies including Alzheimer’s disease and are characterized by deposition of the abnormal tau protein.

Tau proteins are neurospecific microtubule-associated proteins that modulate the stability of axonal microtubules. Modeling of tau behavior in normal conditions and during tauopathy is a challenge by systems biology/pharmacology modeling approaches can lead to a better understanding of the system. Increasing number of literature reports about toxicity of oligomeric forms of tau protein underscore the importance of tau pathologies. Such data is essential in developing tau protein aggregation model to understand the properties of different forms of tau – monomeric, oligomeric, and fibrils.

We developed a mathematical model of ordinary differential equations to describe inducer-dependent tau aggregation. Model describes the following stages of aggregation – conformational transition of tau molecules, nucleation, elongation of tau fibrils and fibril breakage as a secondary pathway for polymer growth. Within the model, monomeric, oligomeric and aggregated forms of tau protein were described. Effect of various inducers on tau aggregation was also investigated within the model. We have found that parameters driving elongation and breakage of tau proteins have the greatest influence of steady-state level of aggregated tau. Different polymerization inducers appear to follow different induction mechanisms for tau aggregation. Amongst the different inducers investigated (arachidonic acid, heparin, and β-amyloid), β-amyloid appears to be the strongest inducer. The model predicts monotonic kinetics of monomeric tau forms and tau fibrils, and bell-shaped kinetics of oligomeric tau forms.

This model enables identification of critical intervention points to impact of potential drugs for clinical management of tauopathies.

**SW04.S19–68**

**Distinct roles of PI3 kinase and MAP kinases in motor neuron regeneration**

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We have developed a novel spinal cord-peripheral nerve co-culture system that closely models adult peripheral nerve injury in vitro. The motor neurons in the spinal cord slices express YFP and the progress of their axons in to the cocultured, non YFP, peripheral nerve can be monitored in real time. Once innervated, the peripheral nerve is transected distally and joined to a fresh nerve graft to simulate nerve injury and repair. We report herein the use of this system to study the signaling events that accompany motor axon injury and regeneration. The intracellular signaling pathways involved in motor neuron regeneration remain poorly understood due to lack of suitable model systems. Herein we report that PI3 kinase and MAP kinases play distinct roles in motor axon initiation, elongation, and the extent of regeneration.
Cognitive decline in Alzheimer’s disease (AD) is usually accompanied by the accumulation of the pathologic amyloid beta (Aβ) protein in brain. We developed a model of Aβ distribution and aggregation in human brain, based on current understanding of biology and existing data from literature.

The model includes distribution of Aβ in 5 compartments (brain cells (BC), brain interstitial fluid (BIF), cerebrospinal fluid (CSF), plasma (PL) and tissue (TS)), and aggregation of Aβ – BIF and BC. It describes the following processes influencing Aβ concentration: release of Aβ in the BIF, BC, TS; transport of Aβ between different compartments; flow of Aβ from BIF to the CSF and from CSF to PL. Description of Aβ aggregation includes the formation and degradation of nuclei that initiate aggregation; formation, breakage and destruction of fibrils; formation of oligomers.

Fitting of parameters for Aβ aggregation was performed using data for Aβ40 and Aβ42 concentration in the brain and CSF for healthy subjects. To describe Aβ concentration data in AD patients, following effect of age on model parameters were proposed: increase of total Aβ release, increase of Aβ42 fraction of production; decrease of destruction of insoluble Aβ.

During the model building process, we assumed that for the maximal difference between AD and healthy patient, positive feedback of Aβ 40 on the total Aβ production, according to literature, was necessary. Intracellular production of Aβ was also important for accurate description of observed data. Model correctly describes the decrease of CSF Aβ concentration in AD patients with increase in insoluble forms of Aβ in brain. One of the interesting predictions is that therapy directed towards activation of Aβ fibril destruction would lead to the increase in CSF concentrations of soluble Aβ.
regenerated axons. GDNF induced phosphorylation of ERK1/2 in spinal cord slices decreased significantly in the presence of PD98059 or U0126. The transected motor axons were severely perturbed and rapidly retracted when JNK kinase was inhibited using SP600125. Motor neuron survival was unaffected, as monitored throughout the course of these experiments. Our results suggest that the PI3K pathway is crucial to initiate regeneration in all motoneurons. Whereas the ERK1/2 MAP kinase pathway functions similarly in most motoneurons, some regenerate normally without its participation.

The SAPK/JNK pathway is critical for the stability of severed axon.

**SW04.S19–72**

**Kinetic model of Ab distribution and aggregation in human**

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Cognitive decline in Alzheimer’s disease (AD) is usually accompanied by the accumulation of the pathologic amyloid beta (Aβ) protein in brain. We developed a model of Aβ distribution and aggregation in human brain, based on current understanding of biology and existing data from literature.

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**SW04.S20 Photoreception and Biochemistry of Vision (IV-S20)**

**SW04.S20–1**

**Harmful bisretinoid side-products of rhodopsin photolysis: age- and pathology-dependence, ways of protection**

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Deficiency of all-trans-retinal removing system leads eventually to the bisretinoid pigment formation. Most of them, including A2E, are lipofuscin granule (LG) fluorophores. LG are able to light-induced generation of reactive oxygen species (ROS) (Ostrovsky et al., 1992; Boulton et al., 1993). LG, isolated from retinal pigment epithelium (RPE) of human cadaver eyes, are more active in the ROS generation than A2E itself. Exposure to light of A2E-containing liposomes leads to release of A2E-oxidized forms into solution (Dontsov et al., 2009). This means that water-soluble oxidized forms of A2E can damage other intracellular organelles (mitochondria, nucleus), and initiate apoptosis.

We have studied fluorescence spectra and LG fluorophore composition in RPE cells isolated from 42 human cadaver eyes. The eyes were obtained from donors of different ages (from 17 to 78 years) (Arbukhanova et al., 2012). In the course of a detailed postmortem examination of the fundus of each eye, it was revealed signs of age-related macular degeneration (AMD) in two of 42 cadaver eyes. The fluorescence maxima of RPE suspensions from the eyes without signs of pathology were 534 ± 4 nm, regardless of the donor age. In case of two eyes with signs of AMD, we have seen shortwave shift to 498 and 509 nm. HPLC analysis has shown almost linear age-related increase of the relative content of A2E and products of its photooxidation and photodegradation. However, the relative content of LG fluorophores extracted from cadaver eyes without and with signs of AMD was different. It can be assumed that the spectral characteristics of fundus autofluorescence under certain pathological states may be different from the norm.
Melanosomes are able to protect the RPE cells from phototoxic A2E and its oxidized forms. Melanosomes unlike LG inhibit the photoperoxidation of cardiolipin liposomes (Dontsov et al., 1999). The synthetic DOPA-melanin and melanosomes from human, bovine and frog RPE are able to bind A2E as well as its oxidized products. When this the antioxidant activity of DOPA-melanin and melanosomes practically does not change (Sakina et al., 2013). In addition to melanosomes oxy-carotenoids can also play a protective role, although they are located in the retina rather than in the RPE. Using Japanese quail Coturnix japonica as a successful model of accelerated aging of human retina we have shown that increased content of oxy-carotenoids in the eyes corresponds to low volumetric content of LG in the RPE cells (Zak et al., 2013).

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SW04.S20–2
Cone visual cycle in the mammalian retina
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Our cone photoreceptors function in bright rapidly changing light conditions and mediate our daytime vision. The critical ability of mammalian cones to quickly regenerate their visual pigment following its destruction by light is made possible by two pathways: the canonical retinal pigment epithelium (RPE) visual cycle which provides chromophore to both rods and cones, and the recently described retina visual cycle which is cone-specific. This pathway is independent of the RPE and instead relies on Müller cells in the neural retina. Unlike the canonical RPE visual cycle which produces 11-cis retinal, the Müller cells convert the all-trans retinol released from photoreceptors following exposure to bright light into 11-cis retinol. This 11-cis retinol then needs to be oxidized into 11-cis retinal before it can be used for pigment regeneration. Our results demonstrate that the retina visual cycle plays a crucial role in daytime vision by extending the functional range of cones to bright light and by driving their initial rapid dark adaptation following exposure to bright light.

The scarcity of cones in the mammalian retina makes molecular and biochemical cone-specific studies extremely challenging. As a result, the mechanisms that enable cones, but not rods, to use this pathway are not understood. We have now identified a mouse strain that resolves this problem – the mutant rd7 mouse lacking a functional rod transcription factor Nr2e3, with hybrid rods with some cone-like morphological features that, in addition, express a subset of cone-specific genes. Our results demonstrate that, similarly to cones and unlike wild type rods, these hybrid rods are able to regenerate their visual pigment and dark adapt without the help of RPE by relying on the retina visual cycle. We conclude that the altered morphology and gene expression of the hybrid rd7 rods enable them to utilize the cone-specific retina visual cycle for pigment regeneration. These mice allow investigating the mechanisms controlling access to the retina visual cycle in the context of the much more abundant rods and hold the key to understanding the specificity of the retina visual cycle.

SW04.S20–3
Rhodopsin diffusion in the photoreceptor membrane
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Current scheme of phototransduction in vertebrate photoreceptors relies on free movement of components of the signalling cascade within the photoreceptor membrane. The mobility is essential for interaction of main proteins and, consequently, for signal amplification and turning-off of the phototransduction. However, recent findings of our lab indicated the existence in intact rods of an immobile (non-diffusing) rhodopsin fraction of greatly varying size (Govardovskii et al., 2009). There could be at least two causes for the formation of the immobile rhodopsin fraction. Firstly, it is suggested that rhodopsin in the photoreceptor membrane exists in a dimeric form, and that dimers may form extended paracrystalline (hence poorly mobile) domains (Fotiadis et al., 2002). Then dynamically changing rhodopsin oligomerization could be an extra way of regulating the cascade. Second, less exciting, explanation of the non-diffusing rhodopsin fraction could be structural restrictions, such as a fragmentation of membrane disks. The fragmentation would form isolated compartments and stop the exchange by rhodopsins between extended disk areas without affecting local processes of phototransduction.

Using high-speed dichroic microspectrophotometer (MSP), we tested effects on the size on the non-diffusible rhodopsin fraction of some agents that are supposed to change the aggregation of membrane lipids and proteins. MSP measurements were done on physiologically intact single rods and single isolated rod outer segments of the frog retina. All tested treatments failed to significantly change the size of the immobile rhodopsin fraction. Then we changed the measuring configuration such a way as to minimize the effect of possible disk fragmentation. In this case we found no evidence of permanently existing oligomeric rhodopsin complexes that would enclose more than 10% of rhodopsin. This makes the idea of a controlled oligomerization less probable.

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SW04.S20–4
New mechanisms of regulatory activity of photoreceptor calcium sensors
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Intracellular calcium signaling with the participation of Ca2+ -sensor proteins is involved in a number of the crucial neuronal functions in health and diseases, among which are reception, neurotransmission, synaptic plasticity, control of neuronal growth, and neuronal survival. In the case of photoreceptor neurons, Ca2+ -sensor proteins are represented by calmodulin and several proteins of the neuronal calcium sensor (NCS) family, including recoverin, guanylyl cyclase activating proteins (GCAPs) and neuronal calcium sensor 1 (NC1). Retina-specific recoverin and GCAPs are considered as the participants of phototransduction involved in the control of rhodopsin phosphorylation and cGMP synthesis, respectively. In contrast to recoverin and GCAPs, calmodulin and NCS1 are widely distributed in the nervous system and whether these proteins perform specific functions in photoreceptor neurons still remains an open question. In this study, we have attempted (i) to answer this question and (ii) to get insight into the mechanism(s), underlying the specificity of the responses of photoreceptor calcium sensors. To this end, we have used Ca2+ -dependent rhodopsin phosphorylation catalyzing by rhodopsin kinase (G-protein coupled receptor kinase 1, GRK1) as a
model of the Ca2+-sensor-mediated process. It has been found that calmodulin increases the magnitude and Ca2+-sensitivity of the well-known inhibitory effect of recoverin upon rhodopsin phosphorylation. This result is in agreement with the existence of separate binding sites for recoverin and calmodulin revealed in the GRK1 molecule by biosensor-based approach. Further, it was demonstrated that NCS1 and GCAP2 interact with GRK1 in a Ca2+-dependent manner. Remarkably, NCS1 acts as inhibitor of the enzyme while GCAP2 apparently does not affect its activity. The roles of the C-terminal segment and the other structural elements of the NCS proteins in the Ca2+-sensitivity and overall specificity of their action are discussed.

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SW04.S20–5

Vascular endothelial growth factor receptor 2 may mediate hydrogen peroxide induced human retinal pigment epithelial cell damage

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Purpose: Reactive oxygen species and the human retinal pigment epithelium (hRPE) have been implicated in the pathogenesis of age related macular degeneration (AMD). Hydrogen peroxide (H2O2), a by-product of oxidative stress, has been shown to stimulate an angiogenic factor, vascular endothelial growth factor (VEGF). However, very little is known about its mechanism of action. This led us to investigate the role of VEGF receptors 1 and 2 (VEGFR1 and VEGFR2) in the response of hRPE to oxidative stress.

Methods: hRPE cultures were established from normal human eyes obtained from the Michigan Eye Bank. Cell proliferation and viability were quantitated by 3H-thymidine (3H-thy) incorporation and by the trypan blue exclusion (T) method. Fetal Bovine Serum (FBS) stimulated cell proliferation was quantitated before and after cell cultures were exposed to anti-VEGFR1, anti-VEGFR2 and anti-FGFR2 in the presence and absence of oxidative stress (H2O2). Statistical significance was determined by Student ‘t’ test.

Results: FBS stimulated hRPE cell growth and cell viability in a dose dependent manner. H2O2 (0.5 mM) exposure prevented the proliferative stimulatory effect of FBS (2.77 ± 0.35 versus 5.66 ± 0.77, viable cells±SEM, n = 6, p ≤ 0.05). H2O2 (0.5 mM)+ anti-VEGFR1 exposure reduced this inhibitory effect increasing the number of FBS simulated viable hRPE cells when compared with H2O2 (0.5 mM) exposure alone (3.0 ± 0.59 versus 1.81 ± 0.34, viable cells±SEM, n = 3, p ≤ 0.05). H2O2 (0.5 mM)+ anti-VEGFR1 resulted in a similar inhibition of stimulatory effect when compared with H2O2 (0.5 mM) alone (2.83 ± 0.39 versus 1.95 ± 0.18, viable cells±SEM, n = 3, p ≥ 0.05), although the inhibition was less dramatic. On the other hand, exposure to Anti-FGFR1 did not increase viable hRPE cell number in H2O2 exposed cells. Phase contrast microscopy confirmed that H2O2 damages hRPE cell morphology.

Conclusion: We have shown that FBS stimulates proliferation of hRPE cells and the oxidative stress of H2O2 inhibits this proliferation. Anti-VEGFR1 and anti-VEGFR2 partially block the H2O2 induced hRPE cell damage, anti-VEGFR2 more effectively than anti-VEGFR1. This inhibitory effect was not seen with Anti-FGFR1. We therefore conclude that VEGF receptors may mediate oxidative damage in hRPE cells and this information may be useful in devising pharmacologic treatment for AMD.

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SW04.S20–6

Inhibition of F0F1-ATPase and ATP synthase by polyphenolic phytochemicals in rod outer segments

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Vertebrate retinal rod Outer Segments (OS) consist of a stack of disks surrounded by the plasma membrane, where phototransduction takes place. Energetic metabolism in rod OS remains obscure. Our previous results from proteomic and biochemical studies on purified bovine OS disks, reported an extramitochondrial ATP synthase activity associated with optical stress (OXPHOS) process. This would largely account for the light stimulated ATP need of the photoreceptor [1]. Here, after a characterization of OXPHOS in the bovine OS by Electron (TEM) and fluorescence microscopy on bovine retina, we show that isolated purified OS synthesize ATP and consume O2 in the presence of uncommon substrates (i.e. citrate, α-ketogluturate, fumarate) suggesting a particular sidedness of the OXPHOS proteins in the OS.

The effect of several natural polyphenolic phytochemicals, known inhibitors of F0F1-ATP synthase, on the ATPase/ATP synthase activity of OS homogenates was investigated. Resveratrol, a stilbene phyoalexin present in grapes and red wine, and curcumin, a principal component of turmeric, inhibited ATP synthase activity of 90% and 20% respectively. Co-administration of curcumin with piperine, a major alkaloid of black pepper, inhibited ATP synthase by 56% showing that known potentiation of curcumin effects by piperine also occurs in vitro. Piperine alone does not exert any inhibitory activity. It may be hypothesized that piperine, besides increasing curcumin bioavailability after oral administration, also play an important role in curcumin mechanism of action. Moreover ATPase activity, tested in purified OS in the presence of ouabain, inhibitor of Na+/K+ATPase, was inhibited of 52% by epigallocatechin gallate, a potent antioxidant catechin found in green tea.

The effect of these phytochemicals on ectopic ATPase/ATP synthase activity in OS are consistent with the hypothesis that OS express a functional F0F1-ATP synthase. Moreover, these antioxidant substances may significantly interact with OXPHOS in the OS, and also scavange reactive oxygen species. This may shed light on retinal pathologies (retinitis pigmentosa and age related macular degeneration) related to oxidative stress [2] and to mutations in TCA enzymes.

References

SW04.S20–7

Comparative analysis of rat retinal transcriptome using RNA-Seq: effects of aging and AMD-like retinopathy

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The leading cause of vision loss in elderly is age-related macular degeneration (AMD), neurodegenerative disease. Environmental, dietary, and genetic factors influence AMD pathogenesis, which
remains poorly understood due to the paucity of animal models. We showed that senescence-accelerated OXYS rats develop a retinopathy similar to human AMD in its clinical and morphological manifestations. To identify alterations in response to normal aging and progression of retinopathy, we compared gene expression profiles of retina from 3- and 18-month-old OXYS and control Wistar rats by RNA sequencing (RNA-Seq). We identified 160 and 146 age-regulated genes in Wistar and OXYS retinas, respectively. The majority of them are related to the immune system and extracellular matrix turnover. Only 24 age-regulated genes were common for the two strains, suggestive of different rates and mechanisms of aging. Over 600 genes showed significant differences in expression between the two strains. These genes are involved in disease-associated pathways such as immune response, inflammation, apoptosis, Ca²⁺ homeostasis, and oxidative stress. The altered expression for selected genes was confirmed by qRT-PCR. To our knowledge, this study represents the first analysis of retinal transcriptome from young and old rats with biologic replicates generated by RNA-Seq technology. We can conclude that the development of retinopathy in OXYS rats is associated with an imbalance in immune and inflammatory responses. Aging had significant effects on the expression of inflammatory genes but their composition was different in the retina of OXYS and Wistar rats. The retinopathy development in OXYS rats accompanied by downregulation of immune response genes in the retina. Individual differences observed with aging occur not only in rodents but also in humans. This indicates that any disturbances in immune defenses can accompany retinal disease, not only upregulation, but also downregulation, which can be explained within the framework of immunosenescence theory. Our data support the view that the genetic background has a profound impact on AMD development and on AMD-like retinopathy in OXYS rats.

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**SW04.S20**

cAMP affects calcium homeostasis and PDE6 in the phototransduction cascade

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The ability of photoreceptor to operate within a wide dynamic range of light intensities is provided by the calcium feedback mechanism. Yet the level of intracellular calcium [Ca²⁺]₀ itself was believed to be set by the same mechanism, via the control of the intracellular level of cGMP. Recently, by measuring the exchange current of frog rod photoreceptors we have shown that [Ca²⁺]₀ might also be affected by an intracellular level of cAMP ([cAMP]₀). That is, elevation of [cAMP]₀ 2.5 times by application of an adenylate cyclase activator, forskolin, evokes 1.6-fold rise of [Ca²⁺]₀ which in turn significantly affects the sensitivity of the photoreceptor by modifying the cascade turnover.

We found also that elevation of [cAMP]₀ results in the decrease of the basal (dark) PDE6 activity which supposedly arises from spontaneous activation and inactivation of the enzyme. We investigated the mechanism of this decrease by analyzing power spectra of the dark current of isolated frog rod photoreceptors using suction pipette. We found that the elevation of [cAMP]₀ affects kinetics of activation/inactivation but not the catalytic properties of PDE6. Apparently, high [cAMP]₀ slows down the spontaneous activation of PDE6. However, the rising phase of the photoreponse remains mostly unaffected by high [cAMP]₀ which means that the maximum catalytic activity of PDE6 stays unchanged. Decreasing of dark PDE6 activity slows down the turnover of cGMP and, like elevation of [Ca²⁺]₀, increases the sensitivity of the photoreceptor. The biological significance of this regulation still remains to be determined.

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**SW04.S20-9**

Unusual photolysis products of a blue-sensitive cone visual pigment in some fish species

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Using a dichroic microspectrophotometer we have found that the photolysis of the visual pigment in blue-sensitive (SWS2) cones in the Cyprinidae (goldfish, carp and zebrafish) and Adrianichthyidae (rice fish Oryzias javanicus) fish is different from the photolysis of other known visual pigments. In all cases, studied till now bleaching of rods and cones produces a mixture of MetaI/MetaII. The mixture exhibits a characteristic absorbance spectrum with a MetaI peak at 380–400 nm and a MetaII shoulder at 470–480 nm. Bleaching of SWS2 in our fish does not generate typical Meta and MetaII. Instead, bleaching produces a long-living product mixture whose spectrum is similar to the ‘dark’ pigment spectrum.

Recordings at two directions of polarization show that post-bleach changes can be described as interconversion and decay of two products that we have named long- (LW) and short-wave-length (SW). The LW- and SW-products look superficially similar to Meta and MetaII, resp., but their absorbance peaks, orientation within the photoreceptor membrane and kinetics of decay differ from ‘standard’ Meta and MetaII. LW-chromophore is aligned parallel to the membrane, like in MetaI, but has absorbance peak at ≈440 nm, i.e., is blue-shifted compared to MetaI. SW-product peaks at ≈390 nm, close to that of MetaII. However, it is orientated across the membrane, while the MetaII chromophore lies strictly within the membrane. Meta in other cone types is a minor component as it converts into MetaII in ≈1 ms. Then MetaI decays in 5–10 s into all-trans retinal and opsin. On the other hand, LW-product is initially presented at high concentration, and lives tens of seconds, partly converting into SW-product. SW-product accumulates up to 30 s post-bleach and then decays in 15–20 min.

Such unusual products are not a specific feature of SWS2-pigments as such, as, for example, SWS2 cones in cichlids Astronotus tiger, Pseudotropheus zebra and Pterophyllum altum exhibit typical MetaI/MetaII mixture. Other visual pigments (SWS1, Rh2, LWS) of the species studied also demonstrate a ‘standard’ photolysis.

The nature of the unusual photoproducts and their effect on the function of SWS2 cones deserves further investigation.

**SW04.S21**

Stem Cells: Fundamentals and Applications (IV-S21)

**SW04.S21-1**

iPS cell technology and disease research: issues to be resolved

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The recent demonstration of in vitro reprogramming using transduction of 4 transcription factors by Yamanaka and colleagues...
Recently, we and others have shown that Nanog is under autoregulation in a gene regulatory network centred on Oct4, Nanog and Sox2. Pluripotent cell identity is governed by the action of apparently symmetrical self-renewing cell division, essentially representing a major advance in the field. However, major questions regarding the mechanism of in vitro reprogramming need to be understood and will be one focus of the talk.

During cellular reprogramming only a small fraction of cells become induced pluripotent stem cells (iPSCs). Previous analyses of gene expression during reprogramming were based on populations of cells and impeded identification of events at the single-cell level. We utilized two different gene expression technologies to profile 48 genes in single cells at various stages during the reprogramming process. Analysis of early stages revealed considerable variation in gene expression between cells in contrast to late stages. We show that Esrrb, Utf1, Lin28, and Dppa2 have a better potential to predict cells that will become iPSCs, compared to Fbxo15, Fgf4, and Oct4, which were previously suggested to be markers of reprogramming. Our data suggest that stochastic gene expression early in reprogramming is followed by a late sequential phase with Sox2 activation upstream in a gene expression hierarchy. Finally, we demonstrate that subsets of downstream factors derived from the sequential phase can activate the pluripotency circuitry.

A major impediment in realizing the potential of ES and iPSC cells to study human diseases is the inefficiency of gene targeting. Using Zn finger or TALEN mediated genome editing we have established efficient protocols to target expressed and silent genes in human ES and iPSC cells. The most recent advance comes from the use of the CRISPR/Cas9 system to engineer ES cells and mice. This technology allows the simultaneous editing of multiple genes and will facilitate establishing relevant models to study human disease.

**SW04.S21–2**

Principles and programming of the germline for totipotency and early mammalian development

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Specification of primordial germ cells (PGCs) in mammals occurs during development of postimplantation epiblast cells, which also give rise to all somatic tissues. We now have wide-ranging knowledge of the mechanism of PGC specification in mice, which is linked to extensive epigenetic reprogramming. PGC specification is followed by sequential, orderly and dynamic epigenetic changes in histone modifications, reactivation of the X chromosome and comprehensive global DNA demethylation. These epigenetic changes are essential towards the establishment of the totipotent state, which follows after fertilisation and establishment of the zygote. Pluripotent state is established subsequently following genetic and epigenetic changes in the zygote and in the course of preimplantation development.

**SW04.S21–4**

Generation of authentic striatal neurons from human pluripotent stem cells for transplantation studies in Huntington’s disease

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Our laboratory works on Huntington’s Disease, an inherited neurodegenerative disorder. On the one hand we are studying the physiology and the evolution of the causative gene (Lo Sardo & Zuccato et al., Nat Neurosci., 2012) as well as the toxicity elicited by its mutant version (Zuccato et al., Physiol. Review, 2010). On the other hand we aim at harnessing the potential of human pluripotent stem cells for the generation of the medium-sized spiny neurons that are affected in the disease. However, the achievement of such a goal requires a more detailed understanding of the biology of human fetal striatal development. Here we will present unpublished data on the spatio-temporal expression pattern of transcription factors that mark human fetal striatal development in vivo. We will also show how incorporation of this information into human pluripotent stem cell differentiation has already allowed, for the first time, the generation of authentic and functionally active DARPP-32+/CTIP2+ medium-sized spiny neurons.

**SW04.S21–5**

Cardiomyocytes from human pluripotent stem cells: the new patient in safety pharmacology, drug discovery and disease

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Derivation of heart cells from human pluripotent stem cells (hPSC) is an area of growing interest as a way of modelling disease phenotypes and as a platform for drug discovery and toxicity. Applying the underlying developmental mechanisms that control cardiac differentiation to hPSCs through the use of defined culture conditions in vitro is rapidly moving the field forward: cardiomyocyte differentiation is now a fairly efficient and reproducible process. Genetically marked hESC have been produced in which expression of the green fluorescent protein marker is driven by specific lineage markers like Nkx2.5. We are now using these tagged lines in which GFP to select the progenitors of cardiomyocytes, endothelial cells and smooth muscle cells. Applications of hESC- and hiPSC-derived cardiomyocytes in...
drug discovery and disease are thus now close to implementation. Results of these studies, in particular drug responses of hPSC-cardiomyocytes to a variety of drugs will be shown. There is an urgent unmet need for reliable cardiac safety pharmacology assays to identify potential risks early in drug development and reduce time and cost to market. The field potential of hPSC-CM can be measured using commercially available multi electrode arrays. Systematic generation of dose response curves for cardiac and non-cardiac drugs show that hPSC-CM accurately predict reported drug effects on the human heart. These include blocking the hERG ion channel, resulting in QT-prolongation; this is associated with life-threatening arrhythmias. Dose responses of a wide range of compounds have been compared and the outcome shown to predict clinical effects.

SW04.S21–6
Transcriptional regulation of thymus regeneration

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The thymus undergoes significant age-related involution, which results in the decreased production of naïve T cells with age and is a major causative factor for age-related decline of immune system function. The epithelial component of the thymic stroma is essential for intrathymic T-cell development and undergoes a stereotypical age-related degeneration that is strongly implicated as a primary cause of involution. The key transcription factor Foxn1 is critically required throughout TEC differentiation in the fetal and postnatal thymus and is down-regulated with age in the thymic stroma, and is also implicated as a target in age-related thymic involution. Whether up-regulating Foxn1 activity is sufficient to drive rejuvenation of the fully involuted, aged thymus however remains unanswered. Indeed, whether the effects of established age-related thymic involution can genuinely be reversed is also unknown. We have tested the outcome of up-regulating Foxn1 expression in the adult thymus using a novel mouse model that permits tissue specific, regulatable Foxn1 expression. Our data establish that forced, TEC-specific, up-regulation of Foxn1 in the fully involuted thymus of aged mice results in robust thymus regeneration characterized by increased thymopoiesis and increased naïve T cell output. We demonstrate that this regeneration stems from an enlarged TEC compartment, rebuilt from progenitor TEC, the architecture and gene expression levels of which are restored to those of the juvenile thymus. Collectively, our data establish that up-regulation of a single transcription factor is sufficient to reverse age-related thymic involution, identifying Foxn1 as a specific target for improving thymus function and thus immune competence in patients.

SW04.S21–7
Induction of pluripotency

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Pluripotent stem cells are characterized by open chromatin and high transcription levels, which is achieved via auto-regulatory and feed-forward transcription factor loops. The reprogramming of mouse and human somatic cells into pluripotent stem cells, designated as induced pluripotent stem (iPS) cells, was first successfully achieved using fibroblasts as the starting population in 2006 by Kazutoshi Takahashi and Shinya Yamanaka. However, it remains unknown how this leads to the multitude of epigenetic changes observed during the reprogramming process. Interestingly, Oct4 is the only factor that cannot be replaced by other members of the same family to induce pluripotency. To understand the unique role of Oct4 in reprogramming, we determined the structure of its POU domain bound to DNA [1]. We show that the linker between the two DNA-binding domains is structured as an α-helix and exposed to the protein’s surface, in contrast to the unstructured linker of Oct1. Point mutations in this α-helix alter or abolish the reprogramming activity of Oct4, but do not affect its other fundamental properties. Based on mass spectrometry studies of the interactome of wild-type and mutant Oct4, we propose that the linker functions as a protein-protein interaction interface and plays a crucial role during reprogramming by recruiting key epigenetic players to Oct4 target genes. Thus, we provide molecular insights to explain how Oct4 contributes to the reprogramming process.

Gene-specific factors for RNA polymerase II-mediated transcription of pluripotency genes recruit transcriptional co-factors and chromatin regulators in order to control access and activity of the basal transcription machinery. Here, we show that TFIIH knockdown affected the pluripotent circuitry in ES cells and inhibited reprogramming of fibroblasts [2]. TFIIH with TAF4 and the pluripotency factors form a feed-forward loop to induce and maintain a stable transcription state. Strikingly, transient expression of TFIIH subunits greatly enhanced reprogramming. These results show that TFIIH is critical for transcription factor-mediated reprogramming.

References


SW04.S21–8
Polycomb complexes co-associate with a specific RNA polymerase II variant in mouse ES cells

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Polycomb repressor complexes are important chromatin modifiers fundamentally implicated in pluripotency and cancer. Polycomb-mediated gene silencing in embryonic stem cells is accompanied by active chromatin marks. Chromatin immunoprecipitation (ChIP) on a small cohort of PRC-repressed genes identified the presence of an unusual form of RNA polymerase II (RNAPII) at promoters and through coding regions, in the absence of active elongation marks (Stock et al. 2007, Nat Cell Biol 9, 1428).

To investigate the relationship between Polycomb and RNAPII genome-wide in ESCs, we mapped several markers of Polycomb repression and four different states of RNAPII activity across the ESC genome using ChIP-seq. We found that PRC-bound genes exhibit a variety of RNAPII states.

SW04 Molecular Mechanisms of Disease
We identify a large cohort of silent developmental Polycomb targets which are bound by unproductive RNAPII (S5p+S7p-S2p-), confirming the earlier single-gene analyses. We find that RNAPII-S5p+ transcribes through coding regions of Polycomb targets without productive expression of functional mRNAs. Based on sequential ChIP between RNAPII and Polycomb components, Ring1B-depletion analyses and genome-wide correlations, we unequivocally show that Polycomb complexes and RNAPII-S5p physically bind to the same chromatin at the same time and functionally synergise.

We currently investigate RNAPII phosphorylation and Polycomb occupancy in neuronal and cardiac lineages.

SW04.S21–9
The interaction between extrinsic signals and intrinsic factors during neural commitment of pluripotent stem cells
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Pluripotent stem cells could differentiate into these three cell lineages, ectoderm, mesoderm and endoderm. During this process, cell fate is determined by integration between extrinsic signals like BMP, Nodal, Wnt, FGF and intrinsic factors, such as transcription factors and epigenetic regulators. To study functions and molecular mechanisms of extrinsic factors in pluripotent stem cell neural differentiation, we established a mouse embryonic stem cell (ESC) neural differentiation protocol and found a BMP-sensitive window during ESC neural differentiation. Cells at this specific period are correspond to the epiblast of the egg cylinder of mouse embryos and can be maintained as ESC-derived epiblast stem cells (ESD-EpiSCs). We further show that BMP signaling has distinct functions in different stages of ESC neural commitment. Using differential screening strategy, we identified two novel downstream targets of BMP signaling pathway, AP2γ and ovol2, which partially mediate BMP functions during cell fate decision of pluripotent stem cells. AP2γ inhibits neural conversion and promotes epidermal development in ESC neural differentiation and at early stages of ectodermal patterning of chick embryo. Ovol2 promotes mesendoderm differentiation and represses neuroectoderm commitment in mouse ESCs and in early chick embryos.

To search for intrinsic factors involved in neural commitment of pluripotent stem cells, we find that Oct6, a class III POU domain transcription factor, is up-regulated during mouse ESC neural differentiation. Oct6 overexpression promotes neural fate determination of mESCs, whereas Oct6-knockdown impairs neural conversion of mESCs. The inducible overexpression or knockdown experiments show that Oct6 does not have impact on ESC differentiating into EpiSCs, but functions specifically in the stage from EpiSCs to neural progenitor cells. In vivo blastocyst injection of Oct6-overexpressing (Oct6-OV) or Oct6-knockdowning (Oct6-KD) ESCs shows the Oct6-OV ESCs prefer to contribute to neuroectoderm but not to other cell lineages in chimeric mice, and Oct6-KD ESCs show the opposite phenotype. These data suggest that Oct6 as an intrinsic factor promotes neural commitment of pluripotent stem cells.

SW04.S21–10
Lgr5 stem cells in self-renewal and cancer
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The intestinal epithelium is the most rapidly self-renewing mammalian tissue. Lgr5 is a gene transcribed in cycling, crypt base columnar cells at the crypt base. Using lineage tracing experiments the Lgr5+ve cells were identified as the stem cells of the intestinal epithelium. Furthermore, Lgr5+ve stem cells can initiate ever-expanding organoids in vitro. The Lgr5+ve stem cell hierarchy of differentiation is maintained in these organoids. Thus, intestinal crypt-villus units can be built from a single stem cell in the absence of a non-epithelial cellular niche.

Although, Lgr5 stem cells persist life-long, crypts drift toward clonality quickly. The cellular dynamics are consistent with a model in which the stem cells divide symmetrically, and stochastically adopt stem or transient amplifying cell fates after cell division.

Lgr5 stem cells are interspersed between differentiated Paneth cells, which produce all essential signals for stem-cell maintenance. Co-culturing of sorted stem cells with Paneth cells dramatically improves organoid formation. Genetic removal of Paneth cells in vivo results in the concomitant loss of Lgr5 stem cells.

Intestinal cancer is initiated by Wnt pathway-activating mutations in genes such as APC. Deletion of APC in stem cells, but not in other crypt cells results in neoplasia, identifying the stem cell as the cell-of-origin of adenomas. Moreover, a stem cell/progenitor cell hierarchy is maintained in stem cell-derived adenomas, lending support to the ‘cancer stem cell’-concept.

SW04.S21–11
Identification of a novel type of immature haematopoietic stem cell (HSC) precursor in mouse development
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Haematopoietic development in vertebrates occurs through the generation of consecutive waves of haematopoiesis; primitive and definitive. The primitive wave is thought to transiently support the immediate needs of the embryo, whereas the definitive wave represents the rudiment of long-lasting adult haematopoietic system. In the mouse, the first definitive hematopoietic stem cells (dHSC), capable of long-term multi-lineage haematopoietic reconstitution of adult recipients, arise in the late E10-E11 aorta-gonad-mesonephros (AGM) region. We previously identified two immediate precursors of dHSC; preHSC type 1 and preHSC type 2, which emerge sequentially in the AGM region of late E10 - E11 embryos.

We have now established a novel in vitro system, which facilitates maturation of dHSCs from the aortic region of the E9.5 mouse embryos. The E9.5 aortic area, placed in these culture conditions can develop dHSCs, which show high level multi-lineage haematopoietic engraftment, following transplantation into adult irradiated recipients. The cell population, enriched for these early precursors of dHSCs, is negative for common hematopoietic marker CD45, but is positive for early hematopoietic marker CD41, key haematopoietic transcription factor Runx1 and can generate both committed progenitors and dHSCs.
Poly(ADP-ribose) acts as a signaling molecule during hydrogen peroxide-mediated osteogenic differentiation
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The attachment of NAD+-derived (ADP-ribose) to proteins, called poly(ADP-ribose)ation, facilitates cell response to DNA damage, and is also involved in cellular signaling. We investigated the role of poly(ADP-ribose)ation during osteogenic differentiation using the SAOS-2 and mesenchymal stem cells. Upon exposure to osteogenic medium both cell lines revealed osteoblast-like features: calcium deposition, increased alkaline phosphatase activity, gene expression of osteoblastic markers (Runx2, BMP2), and commitment to death (apoptosis and necroptosis). Catalase reduced the differentiation and cell death in both cell types. The release of hydrogen peroxide and accumulation of oxidative damage of cellular components correlated well with the level of poly(ADP-ribose)ose, synthesis of which was also delayed by catalase. The silencing of the main poly(ADP-ribose) synthesizing enzyme PARP-1 and polymer degrading enzyme poly(ADP-ribose) glycohydrolase (PARG) (shPARP-1 and shPARG cells, respectively) resulted in enhanced or suppressed cellular PAR levels, respectively. Both shPARP-1 or shPARG cells underwent differentiation, however, most parameters of osteogenic differentiation was delayed in shPARG-1 cells. While PARP-1 silencing suppressed apoptosis and necroptosis, inhibition of the enzyme with the specific inhibitor PJ34 augmented cell death indicating that the effect of PARP-1 on osteogenic differentiation is – at least in part – independent of its enzymatic activity. PARG silencing, which led to PAR accumulation promoted cell death, although translocation of apoptosis inducing factor from the mitochondria to nucleus was not observed. Our study proves that hydrogen peroxide-mediated redox signaling requires the concerted action of PARP-1 and PARG in osteogenic differentiation and accompanying cell death

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Histone acetylation reduce differentiation and neovascularization potential of endothelial progenitor cells in vitro
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Epigenetic mechanisms regulate gene expression patterns affecting cell function and differentiation. Neovascularization depends on the activation/inactivation of genes associated with proliferation, adhesion, migration, and invasion of endothelial progenitor cells (EPCs). In this study, we examine the role of histone acetylation in differentiation and neovascularization potential of EPCs. Endothelial progenitors cells were treated with the histone deacetylase inhibitor Trichostatin A (TSA), and subjected to morphological, gene expression and functional tests. The level of histone acetylation was highlighted by western blot assay. Quantitative RT-PCR showed that TSA down-regulated the expression of endothelial genes involved in adhesion and angiogenesis such as VE-cadherin, CD133, ICAM-1, Tie-2, VEGFR-2. Furthermore flow cytometry analysis illustrated that TSA reduce the expression of surface markers CXCR4, CD31, CD133, CD117, VEGFR-2. MTT and PCNA (proliferating cell nuclear antigen) assays shows that under acetylation state EPCs proliferation and invasion was decreased. Also, migration and capacity to form capillary tube-like structures in vitro was diminished after treatment with TSA. In conclusion epigenetic mechanism, such acetylation plays an important role in stem cell differentiation, understanding these mechanisms will improve stem cell applications for tissue regeneration therapies.

Effects of GDNF and its synthetic modifications to the nerve cells
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GDNF is a major factor for a survival of the dopamine neurons of the midbrain. It supports the axon growth as well as survival of the neurons of that type. For the different models of the Parkinson disease it has been shown that GDNF could prevent the neurotoxically provoked death of the dopamine neurons and supports recuperation of its functional activity. Though some by-side effects are also known, like loosing of weight and possibility of neoplastic transformation. We prepared a genetic construct caring human GDNF, introduced it into HEK293 cells, and then transplanted the cells into parenchyma of the mouse brain. It has been shown that transgenic cells which express GDNF essentially reduce the glial scar formation. Therefore GDNF could be applied during transplantation into the brain to improve the transplant survival.

Though in humans GDNF gene supply two versions of mRNA: pre-(α)-pro-GDNF and truncated pre-(β)-pro-GDNF. Pre-(α)-pro-GDNF is secreted through Golgi apparatus and pre-(β)-pro-GDNF is located in the secretory vesicles and moves by fast secretion pathway. It is possible that pre-(α)-pro-GDNF is needed for conventional neuron survival, and pre-(β)-pro-GDNF serves as SOS system during traumatic injury of neurons or neurodegenerative diseases. To study ‘pro’ region function during fast transport and factor induction properties several versions of modified GDNF were made. A secretion of the factor into medium has been shown by western blot analysis. All modified GDNF were introduced into HEK293 cells, and transgenic cell lines were maintained. The condition media after culturing the cells with modified GDNF was added into culture medium of rat embryonal spinal ganglion explant and a growth of neural sprouts were analyzed. Deletion of ‘pro’ region essentially increases of GDNF effects as neural inducer. A study of culture of dissociated spinal ganglion and calculation of neural sprouts yielded the same results.

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Human artificial chromosomes for regenerative medicine and gene therapy
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Studying molecular mechanism controlling pluripotent stem cell self-renewal and differentiation is a highly relevant pursuit, however, there is a clear need to develop novel approaches that would ensure rapid and safe introduction of these unique cells to...
clinics, beneficial for human health. The most recent and exciting topic of our research aimed at the clinical application is one dedicated to a tumor-free use of embryonic stem (ES) and induced pluripotent stem (iPS) cells in tissue-replacement therapies. We will bolster a genetic sensitization method previously developed by us by putting it on the non-integrative platform. This modification should basically eliminate the risks of both insertional mutagenesis by suicidal DNA-cassette, as well as epigenetic silencing of this cassette following its genome integration. We intend to achieve this goal by deploying the human artificial chromosomes (HACs), and further extent the approach, which will combine ES/iPS-based tissue-replacement, genetic sensitization, and gene therapy technologies, onto treatment of recessive hereditary diseases.

SW04.S21–16
The variant histone H2A.Z is a general facilitator of chromatin remodeling
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The replacement variant histone H2A.Z has been implicated in a wide variety of functions, in particular: (i) inhibiting the spread of heterochromatin; (ii) in gene activation (from its location at the TSS of active genes); (iii) in gene repression (from its location at the TSS of repressed bivalent genes); (iv) in the indirect control of gene expression (from its location at enhancers and insulators). To help resolve this conundrum we used ChIP-Seq for genome-wide mapping of total H2A.Z and acetylated H2A.Z in the TSS of both active and bivalent genes, in most cases in the acetylation state, co-mapping with H3K4me3. Enhancers and CTCF binding sites also carry H2A.Z. Knockdown (KD) of H2A.Z in mouse embryonic stem cells (mESCs). H2A.Z was found at the TSS of both active and bivalent genes, in most cases in the acetylated state, co-mapping with H3K4me3. Enhancers and CTCF binding sites also carry H2A.Z. Knockdown (KD) of H2A.Z in mESCs led to reduced expression of pluripotency genes such as Sox2 and Klf4 – implying a role in support of pluripotency – as well as up-regulation of several differentiation-specific (bivalent) genes – implying a role in their repression. The reasons for such a complex phenotype are not immediately apparent from these data, however one hint as to function came from noting that KD of H2A.Z (i.e. its replacement by H2A) leads to decreased nucleosome accessibility at promoters and enhancers – an observation consistent with the known reduced stability of H2A.Z-containing nucleosomes. Another clue was provided by the finding (Yang et al., PLoS Genetics, 8, e1002604) that when hypermethylated repressed genes are reactivated with treatment with 5-azac, an early step is the deposition of H2A.Z into nucleosomes at their promoters. All these data are consistent with a model in which H2A.Z-containing nucleosomes facilitate chromatin remodeling that enables the binding of a diverse variety of complexes – as required for active transcription and its enhancement, or for gene repression. Other DNA transactions, such as repair and replication, may also need deposition of H2A.Z.

SW04.S21–17
SIK2 involvement in downregulation of FGF signaling through Gab1 and Raf1
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Fibroblast Growth Factor 2 (FGF2) is known to stimulate Müller cell proliferation. The signal is propagated via Ras/MAPK pathway and involves rapid and transient ERK1/2 activation. Results from our laboratory suggest salt inducible kinase 2 (SIK2) contributes to the regulation of FGF signaling via serine/threonine phosphorylations of pathway elements.

Among the FGF pathway elements, Gab1 and Raf1 have the canonical SIK2 phosphorylation motif. Therefore, we first aimed to investigate whether SIK2 targets Ser266 on Gab1 and Ser621 on Raf1. These serine residues were mutated to alanine via site-directed mutagenesis. As Ser621 has been described as an autophosphorylation site, Raf-1 was also rendered kinase inactive. Wild type and mutant proteins were expressed in HEK 293T cells, purified by immunoprecipitation and used in in vitro kinase assays. Results obtained indicated that these mutations obliterated the phosphorylation of these proteins by SIK2, thus verified that SIK2 targets these serine residues. In a retinal Müller stem cell line, MIO-M1, co-immunoprecipitation studies revealed that S266A mutation increases FGF dependent Gab1-protein tyrosine phosphatase non-receptor type 11 (Shp2) binding, no differences were evident in Grb2-Gab1 interaction. Therefore, it is conceivable that the Ser266 phosphorylation by SIK2 is important in transient nature of Gab1 interaction with Shp2 and might regulate FGF-dependent ERK activation. Furthermore, Ki-67 immunostainings showed that S266A mutation led to a modest increase in FGF dependent proliferation of MIO-M1 cells compared to wild type Gab1

Our findings support the hypothesis suggesting SIK2 is involved in negative feedback regulation of FGF pathway via phosphorylations of Ser266 on Gab1 and Ser621 on Raf1.

SW04.S21–18
Mesenchymal stem cells expressing cytosine deaminase inhibit growth of murine melanoma in vivo
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The aim of our study was to estimate the efficacy of mesenchymal stem cell-based suicidal gene therapy in mice bearing murine melanoma B16. Adipose mesenchymal stem cells (MSCs) were transfected with plasmid constructs expressing cytosine deaminase (CDA) or cytosine deaminase fused with HSV-1 tegument protein VP22 (CDA/VP22). CDA converts a relatively non-toxic 5-fluorouracil (5-FU), whereas VP22 protein is known for its ability to facilitate secretion and re-uptake of fused proteins to neighbouring cells. We have previously shown the cytoxic activity of CDA-MSCs on murine Lewis lung carcinoma and melanoma B16F10 cells in vitro [1]. In this study, we demonstrate that direct intratumoral transplantation of MSCs expressing CDA or CDA/VP22 followed by systemic administration of 5-FU results in a significant tumor growth inhibition (TGI). There was 53% reduction in tumor volume in mice treated with CDA-MSCs and 58% reduction in tumor volume in mice treated with CDA/VP22-MSCs as compared with control animals transplanted with B16 melanoma alone. The mean tumor doubling time (Td) of B16 melanoma in exponential phase of growth in control animals was found to be 2 days, while Td of treated groups was about 3 days. Injection of CDA-MSC and CDA/VP22-MSC prolonged the life span of mice bearing B16 melanoma by 14% and 28%, respectively. Combination of MSC transplantation with chemotherapy by lysomustine, a member of alkylnitrosourea family of anticancer drugs, resulted in overall potentiation of antitumor effect, with TGI increased to 79% and animal life span to 37%. Our data indicate that in murine B16 melanoma model, MSCs encoding CDA suicide gene
have significant antitumor effect, which becomes even more pronounced when used in combination with lymosustine.

References

SW04.S21–19
The cleavage of Laminin-111 by MMP-2 affects early differentiation of murine ESCs and iPS cells
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Embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells are the most promising cell types in tissue engineering providing an infinite source of different cell types. However, the use of stem cells in medicine is still hindered by a lack of basic understanding of the early onset of differentiation and fate. Laminin-111 (LN-111) is among the first extracellular matrix proteins expressed during embryogenesis [1]. Thus, LN-111 is one of the key players in early differentiation and a deeper understanding of the interplay between LN-111 and stem cells will lead to a better insight into early stem cell fate. Matrix metalloproteinase-2 (MMP-2) is highly up-regulated during the first days of ESC and iPS cell differentiation and processes a variety of different extracellular matrix proteins [2]. Here we show that MMP-2 cleaves off a LN-111 domain leading to a breakup of the LN-111 network [3]. Additionally, this fragment is released as a soluble factor and becomes available for cell surface receptors, where the impact on murine ESCs and iPS cells is discussed. The specific degradation of the LN-111 network by MMP-2, which starts being active at the beginning of ESC and iPS cell differentiation as well as the effect on cell fate gives us insight in the mechanisms guiding early stem cell fate.

References

SW04.S21–20
Inhibitory effects of mesenchymal stem cells on lymphoblastic leukemia cell proliferation
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Mesenchymal stem cells (MSCs) represent a new approach to the treatment of several neoplastic or non-neoplastic disorders. Their potential to repair damaged tissues through transdifferentiation in conjunction with their immunomodulator ability made them promising candidates for cell-based immunotherapy and regenerative medicine. In the present study, we aimed to determine the effects of MSCs on proliferation, apoptosis and gene expression profile of the Acute Lymphoblastic Leukemia (ALL) cell line CCRF-CEM. The experiments were performed after MSCs and CCRF-CEM cells were co-cultured for 72 hours. We analyzed the gene expression patterns to predict oncogenic pathway dysregulation in the cell groups by quantitative RT-PCR and immunohistochemical staining. Mono and oligonucleosomes were detected and Annexin V-FITC/PI staining was performed to measure apoptotic cell death in the cell systems. Using this approach, cell proliferation was significantly inhibited in co-cultured CCRF-CEM cells compared to the control. Furthermore growth factors, p53, Bax and Caspase-9 expressions were increased and cell-signaling gene expressions decreased significantly. Our results clearly showed that, p53 re-expression could be provided by MSC cell derived factors in CCRF-CEM cells. Bax protein, which can directly activated by p53, also increased significantly in the co-cultured CCRF-CEM cells and most likely increased expressions of p53/Pa induced the apoptosis in these cells. In conclusion, we have shown that MSCs have inhibitory effects on proliferation of the ALL cells. Apoptosis was induced by p53-Bax dependent manner and expression profile of genes associated with angiogenesis and cell signaling pathways was dysregulated after co-culture of ALL cells with MSCs. These results open prospects for cellular therapy by MSCs in ALL and could be extended to the next generation of stem cell therapeutics.

SW04.S21–21
Fluorescent bioimaging in the study of the different models ‘stem cells-tumor’ interaction
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It is known, that mesenchymal stem cells play important roles in tumor pathogenesis. For this reason understanding of interaction between stem cells (SC) and tumors is the subject of intense study. We investigated various models of tumor and stem cells interaction using the methods of in vivo fluorescent imaging and confocal laser scanning microscopy (LSM).

In our work we used two different models. In the first model the objects of research were nude mice with transplanted tumor HeLa Kyoto (human cervical carcinoma). Adipose-derived adult human stem (ADAS) cells transfected with the gene of fluorescence protein Turbo RF-P635 were administrated into the experimental animals at different stages of tumor growth (0–8 days) intravenously or into tumor. In the second model bone marrow stem cells taken from GFP transgenic mice C57Bl6 were administrated in subletal irradiated mice C57Bl6 with transplanted Lewis lung carcinoma.

In the first model in vivo imaging has shown unambiguous localization of red fluorescent labeled SC in animals spleen of different groups on day 5–9 after injection. Comparing with LSM data in the case of i.v. injection of ADAS, bright fluorescent structures with the spectrum of protein Turbo RF-P635 are locally accumulated in the bone marrow, lungs and tumor tissues of animals. In the second model internal organs and tumor tissues of animals were investigated at different time after injection of labeled GFP stem cells by the LSM. We identified fluorescent structures with GFP spectral characteristics in lungs, spleen, liver and tumor tissues of animals.

These findings indicate, that stem cells integrate in the animal organism with transplanted tumor and can be identified by the in vivo fluorescent bioimaging techniques and LSM.
SW04.S21–22
Effects of mobilization with G-CSF and apheresis processes on inflammatory markers in healthy voluntary donors
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Allogenic hematopoietic stem cell transplantation is a technique which is used to treat patients who have malign or nonmalign disorders. This study was conducted with healthy donors of 25 patients who applied to Gazi University Medical Faculty Stem Cell Transplantation Unit. Sera of healthy donors whose haematopoietic stem cells were mobilized (separated from bone marrow and entered into circulation) with granulocyte colony stimulating factor (G-CSF) were used to investigate the effects of mobilization with G-CSF and apheresis processes on inflammatory markers. In our study, we administered 10 μg/kg/day G-CSF for 4 days to healthy donors and after apheresis process acquired 4.11 × 10⁶ CD34+ cells/kg average. Serum samples were used to investigate ferritin(ng/ml), IL-6(pg/ml), hs-CRP(mg/dl), levels respectively. Parameters were given as median (min-max). * p < 0.05 compared to basal level

Before G-CSF mobilization (Baseline): 49.4 (2.7–435.4), 6.35 (0.64–85.64), 0.20 (0.00–0.51)
4th day of G-CSF mobilization (before apheresis): 107.2 (34.6–653.3), 7.07 (0.64–94.21), 0.34 (0.00–0.54)
Immediately after apheresis: 193.3 (27.1–528.4), 7.07 (2.07–82.78), 0.34 (0.00–0.54)
24 hours after apheresis: 126.2 (41.1–610), 8.78 (7.28–75.64), 0.47 (0.05–0.60)
1 week after apheresis: 1100.2 (5.2–912.5), 3.51 (3.15–57.07), 0.13 (0.00–0.47)

These data showed that there was a statistically nonsignificant increase IL-6 levels at the 4th day of mobilization with G-CSF, after apheresis and 24 hours after apheresis process and a significant decrease 1 week after apheresis, compared to basal levels. hs-CRP levels were found to be significantly elevated at the 4th day of mobilization with G-CSF, after apheresis and 24 hours after the process compared to basal levels. However, hs-CRP levels decreased to basal conditions 1 week after apheresis. Ferritin levels of the donors were found to be significantly high at all stages compared to initial levels. Our results showed that mobilization with G-CSF and apheresis caused temporary inflammation in healthy peripheric stem cell donors. Furthermore this process affected iron metabolism. We believe that the results should be validated with a larger sample size and a longer follow up.

SW04.S21–23
Proangiogenic features of umbilical cord matrix-derived mesenchymal stromal/stem cells and their ability to function as perivascular-like cells
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Mesenchymal stromal/stem cells derived from human umbilical cord matrix, Wharton’s jelly (WJ-MSC) have emerged as a favorable source for autologous and allogenic cell therapy. Here, we characterized the proangiogenic features of WJ-MSCs and examined their ability to form functional vessels in in vivo models. First, we examined whether WJ-MSCs express endothelial and smooth muscle cell specific markers after culture in endothelial growth media. WJ-MSCs expressed an endothelial specific marker, VEGFR1, at mRNA and protein levels, but did not express other specific markers (VEGFR2, Tie2, vWF, CD31, VE-cadherin). Rather, WJ-MSCs expressed smooth muscle cell specific markers, α-SMA, PDGFβ-β and calponin, and were unable to form tube-like structures with lumens on Matrigel. WJ-MSCs secreted growth factors including angiogenin, IGFBP-3, MCP-1, and IL-8, which stimulated endothelial proliferation, migration, and tube formation. When WJ-MSCs suspended in Matrigel were implanted into nude mice, it led to formation of functional vessels containing erythrocytes after 7 days. However, implantation of endothelial cell-suspended Matrigel resulted in no perfused vessels. The implanted WJ-MSCs were stained positively for calponin or PDGFRβ and were located adjacent to the lining of mouse endothelial cells that were stained with labeled BS-lectin B4. In a murine hindlimb ischemia model, the transplantation of MSCs into the ischemic limbs improved perfusion recovery and neovascularization of the limbs compared to control group. Therefore, the results suggest that WJ-MSCs promote neovascularization and perfusion by secreting paracrine factors and by functioning as perivascular-like cells.

SW04.S21–24
NIH 3T3 cell lines supporting hematopoietic progenitors: in search of factors maintaining hematopoietic stem and progenitor cells ex vivo
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Hematopoietic stem cells (HSC), unlike many other stem cell types, cannot be efficiently maintained and expanded in culture today. Identification of factors that could ensure stable expansion of HSC would be of paramount importance for bone marrow transplantation, gene therapy and regenerative medicine. As suggested by several studies, activation of Notch signaling pathway might be operative in HSC support. To investigate this, we retrovirally expressed Notch ligand Jagged1 in non-supporting murine fibroblast line NIH 3T3. Unexpectedly, the level of hematopoietic support did not correlate with expression of Jagged1 protein in selected clones. To identify additional factors of supporting activity, we performed global analysis of gene expression in studying clones using Affymetrix arrays and found several dozen of differentially expressed genes. Among these genes are Sfrp1, an antagonist of Wnt signaling, and CXCL12 which plays an important role in different aspects of HSC functions. Experiments to verify expression patterns of the found genes by Real Time PCR showed that in hematopoiesis-supporting clone expression levels of Sfrp1 and CXCL12 genes are downregulated and upregulated, respectively. According to these data we can suggest that in supporting clones Jagged1 might cooperate with several identified genes with significantly changed expression levels making these clones hematopoiesis-supporting ones.
Biodegradable scaffolds are essential tools for tissue regeneration. Collagen and elastin are widely used as they are the main components of extracellular matrix. We have incorporated gelatin in the polymeric construct forming thin films. These components were combined at different ratios and polymerized on tissue culture flasks. Human mesenchymal stem cells were grown on the films. As revealed by MTT test films had no toxicity and were suitable for cell culturing. Differentiation was induced by chondrocyte differentiation agents and later tested for extracellular matrix formation as well as chondrocyte and stem cell specific markers such as Sox-9 and Notch-4. Proper chondrocyte formation was also observed by histochemical methods.

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Natural killer (NK) cells are a subset of lymphocytes crucial for innate and adaptive immune responses. NK cells are differentiated from hematopoietic stem cells (HSCs) in the bone marrow (BM). The precise mechanisms, however, governing NK cell development remain unclear. Here we show a stimulatory effect of cryptotanshinone and tanshinone, isolated from Salvia miltiorrhiza Bunge, on the differentiation of NK cells from hematopoietic stem cells (HSCs) in vitro. In the presence of IL-15, tanshinones increased NK cell differentiation, NK cell maturation and the expression of several transcription factors, including Id2, GATA3, T-bet, and Ets-1. Additionally, tanshinones increased p38 MAPK phosphorylation during NK cell differentiation. Furthermore, the p38 inhibitor SB203580 blocked the developmental effects of the tanshinones and suppressed Id2, T-bet, and Ets-1 expression during NK cell differentiation. These results suggest that tanshinones significantly increased IL-15-induced NK cell differentiation via enhancing the p38 phosphorylation and the expression of transcription factors.

Collagen-elastin based films as scaffolds for mesenchymal stem cells

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SW04.S21–26
Cryptotanshinone and tanshinone promote natural killer cell differentiation from hematopoietic stem cells

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Cryptotanshinone and tanshinone promote natural killer cell differentiation from hematopoietic stem cells

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Mouse embryonic stem cells carrying human artificial chromosome

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Human artificial chromosomes (HACs) are a powerful DNA vector system developed recently to introduce large chromosomal fragments, genes and regulatory elements into cultured mammalian cells without affecting the host genome. This approach is devoid of known problems of viral or other vector tools such as insertional mutagenesis and unstable expression. However, the delivery of HACs directly into cells of living organism is feasible so far only via cultured cells that are first to be targeted with HACs, and then incorporated into desired tissues and organs. Pluripotent stem cells such as embryo-derived ES cells and autologous iPS cells seem to be an ideal choice for HAC delivery via tissue-replacement because they possess the capacity for unlimited self-renewal ex vivo and can differentiate into virtually any cell type of the organism both in vivo and in vitro. Our final aim is to develop HAC-based approaches for combined gene and tissue-replacement therapy, which will eventually allow tackling a broad spectrum of recessive hereditary diseases. In this study we report a critical step of this strategy, namely the transfer of HACs into of embryonic stem cells. We successfully transferred aphioid-tetO-HAC, expressing EGFP, into mouse ES cells. Autonomous of HAC was checked via FISH analysis of chromosome spreads. Also we checked pluripotent state of HAC-bearing ES cells by the analysis of expression of pluripotency markers, teratoma-formation test, and via direct differentiation assays. Our results imply that the presence of HACs does not affect the basic properties of ES cells and that the latter cells can be successfully used as a tool for delivery of gene therapeutic HACs into tissue and organs of adult body.

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Lipin 1 coactivates PPARγ transcriptional activity

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Peroxisome proliferator-activated receptor-γ (PPARγ) is a master transcription factor involved in adipogenesis through the regulating adipocyte-specific gene expression. Lipin 1 is known to act as a key factor for the adipocyte maturation and maintenance with modulating C/EBPα and PPARγ network. The present study revealed that lipin 1 activates PPARγ by releasing corepressors, nuclear receptor corepressor 1 (NcoR1) and silencing mediator of retinoid and thyroid hormone receptors (SMRT), from PPARγ in the absence of ligand, rosiglitazone. Also, we identified a novel lipin 1 transcriptional activation domain (TAD), between residues 217 and 399, which is critical specifically to activate PPARγ. Furthermore, this TAD is unique to lipin 1. The physical interaction between lipin 1 and PPARγ occurs at the lipin 1 C-terminal region from residues 825–926, where the VXVXL motif at residue 885 is critical for binding with and the activation of PPARγ. The action of lipin 1 as a coactivator of PPARγ enhanced the adipocyte differentiation, where the TAD and VXVXL motif played the critical role but the catalytic activity of lipin 1 was not directly involved.

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Objectives: CCN family member 2/Connective tissue growth factor (CCN2/CTGF) is a multi-potent factor in proliferation, differentiation and migration of mesenchymal cells. We and other
researchers reported that CCN2/CTGF bound to cytokines and receptors and modified their signaling. We comprehensively sought additional factors binding to CCN2/CTGF and found that receptor activator of NF-kappa B (RANK) as a potential CCN2-binding partner. RANK-RANK ligand (RANKL) signaling is indispensable for the osteoclastogenesis. In the present study, we investigated the role of CCN2/CTGF in RANK-RANKL system to clarify the role of CCN2/CTGF in osteoclastogenesis.

**Methods:** We used a phage-display system to specify amino sequences binding to CCN2. Multiple molecular interactions were evaluated by solid-phase binding assays. The affinities of the bindings were calculated by using a surface plasmon resonance (SPR) analyzer. Nuclear translocation of NF-kappa B was monitored by fluorescence microscopy and was quantified using image analysis software. The activation of MAPK was detected by western blotting. The differentiation of osteoclast was monitored by TRAP staining.

**Results:** CCN2 directly bound to RANK and OPG via a common domain of CCN2. CCN2 enhanced the RANK-mediated signaling such as NF-kappa B, ERK and JNK pathways. The binding of RANK-RANKL was not affected by CCN2; however CCN2 prevented the inhibitory effect of osteoprotegerin (OPG) on differentiation of osteoclast. Of note, OPG inhibited the binding of CCN2 to RANK.

**Conclusion:** CCN2 directly bound to RANK and enhanced RANK signaling induced by RANKL. Moreover, CCN2 also bound to OPG and decreased its inhibitory effect on osteoclastogenesis. Additionally, the fact that OPG inhibited the binding of CCN2 to RANK suggested that OPG inhibited not only RANK-RANKL binding but also RANK-CCN2 binding.

**SW04.S21–30**

The mood stabilizers valproate and lithium activate human FGF1 gene promoter through inhibition of HDAC and GSK-3 activities

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Valproic acid (VPA) and lithium chloride (LiCl) are two primary mood-stabilizing drugs to exert neuroprotective effects and to treat bipolar disorder in clinic. Fibroblast growth factor 1 (FGF1) has been shown to regulate cell proliferation, cell division and neurogenesis. Human FGF1 gene 1B promoter (–540 to +31)-driven green fluorescence (F1BGFP) has been shown to recapitate endogenous FGF1 gene expression. It could also be used to isolate neural stem/progenitor cells (NSPCs) from developing and adult mouse brains as well as human brain tissues. We have previously shown that transcription factors RFX1, RFX2 and RFX3 could directly bind the 18-bp cis-element (–484 to –467), and contribute to the maintenance of F1BGFP(+) NSPCs. In this study, we provide several lines of evidence to demonstrate the underlying mechanisms of VPA and LiCl in activating FGF1-1B promoter activity: (i) VPA and LiCl significantly up-regulated the F1BGFP expression; (ii) the induction of F1BGFP expression involves changes of RFX1-3 transcriptional complexes on the 18-bp cis-element of FGF1-1B promoter; (iii) VPA and LiCl significantly increased the expression levels of FGF1-1B, RFX2 and RFX3 transcripts; (iv) treatments of other HDAC inhibitors, sodium butyrate and trichostatin A, or transfection with GSK-3 siRNAs also activated FGF1-1B promoter; (v) VPA specifically enhanced neuronal differentiation in F1BGFP(+) NSPCs rather than GFP(+) cells. This study suggested, for the first time, that FGF1 is an important target for the mood stabilizers, VPA and LiCl. Our results provide valuable implications of VPA and LiCl in the treatment of bipolar disorder.

**SW04.S21–31**

Expression of Sox2 and Oct4 in normal and diabetic human term placentas

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Placenta has several functions on behalf of healthy embryonic development, besides a good source of stem cell. Both amniotic and chorionic mesenchymal stem cells are isolated from term placentas. Sox2 plays key role in maintaining self-renewal, or pluripotency of undifferentiated stem cells. Oct4 is also used as a marker for undifferentiated cells. It is reported that both amniotic and chorionic mesenchymal stromal cells express Oct4, Sox2 mRNAs in human term placenta.

Diabetes mellitus is a metabolic disorder that affects millions of people worldwide. In diabetic pregnancies several placental disorders may adversely affect intrauterine life. Therefore we wanted to test whether abnormal placentation growth in diabetic patients will be associated with placental stem cells or not. We analyzed Oct4 and Sox2 expression in human term placental by Western Blot and Immunohistochemistry. We found that Oct4 and Sox2 expression was positive at human term placenta. Oct4 protein level decreased in diabetic placentas but Sox2 level was similar. According to our immunohistochemistry results, Oct4 and Sox2 were positively stained in chorionic mesenchymal stromal cells. Immune reaction densities were similar between the groups. Interestingly, Oct4 and Sox2 were also positive at syncytiotrophoblasts.

Although these proteins are pluripotency markers, Oct4 and Sox2 might have different roles in different tissues and cells. Pathologies in diabetic placentas will be related with abnormal cellular proliferation/differentiation mechanisms including placental stem cells. It is worth to study the pluripotency of placental stem cells in pathological conditions in detail, because it could be the main reason lying underneath the abnormal processes.
expressed the stem cell markers Oct-4, Nanog, Sox-2 and nestin while after neural differentiation cells became positive to the neuronal markers NeuN and MAP2.

Differentiated cells derived from NPC patients showed a massive lysosomal accumulation of cholesterol and a percentage of these cells accumulated GM2 ganglioside as well. A morphometric analysis showed that NPC differentiated cells presented longer neurites and a greater number of neurite extremities, segments, roots and node points than normal control derived cells. Thus, cells from NPC patients displayed characteristic features of NPC disease and seem to recapitulate what has been described in human cortical NPC neurons.

In conclusion, we generated a human neuronal model of NPC disease through the induction of differentiation of stem cells obtained from patient’s easily accessible sources.

This strategy may be applied to other neurodegenerative diseases and might represent a powerful tool to perform drug screening on cells obtained from NPC patients.

**SW04.S21–33**

Premature senescence and apoptosis are different responses of human embryonic stem cells, their differentiated progeny and adult stem cells to sublethal stresses

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The cells damaged during their lifespan should have mechanisms to preserve their initial properties. Whether these mechanisms are similar in different cells is under debate. In our research pluripotent human embryonic stem cells (hESC), their differentiated progeny fibroblast-like cells (FLC) generated by spontaneous hESC differentiation and endodermic mesenchymal stem cells (eMSC) were used. The purpose of the work was to investigate genotoxic and proteotoxic stress response of hESC, FLC and eMSC. Sublethal heat shock and doxorubicin treatment were used as stressor factors. It was found that under equal sublethal stress conditions hESC underwent apoptosis whereas FLC and eMSC did not display reduced viability. However, FLC and eMSC subjected to both the heat shock and doxorubicin treatment exhibited features of stress-induced premature senescence (SIPS). It was demonstrated that sublethal heat shock and doxorubicin induced irreversible cell cycle arrest in both FLC and eMSC. These cells were arrested in G0/G1 and G2/M cell cycle phases. The cells had altered morphology, exhibited SA-β-gal activity and increased p21 expression. Neither SA-β-gal-positive cells nor changes in the cell cycle progression were observed in cultures of stressed hESC. To investigate whether the sublethal stress had irreversible impact on proliferation of hESC, 4 days after treatment, cells were replated and cultivated during several passages. hESC survived the sublethal stress sustained renewal, preserved the expression of pluripotent markers and capacity to differentiate into three germ layer cells. The progeny of eMSC and FLC survived stress treatment also retain the properties of their parental cells: they were diploid, had limited lifespan in culture and expressed specific surface markers. Our finding indicate that differentiated progeny of hESC and adult stem cells have similar mechanisms of stress defense but different from those of hESC. Equal sublethal stress induced hESC apoptosis and SIPS in FLC and eMSC. The cells survived stress sustain the properties of parental cells.

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**SW04.S21–34**

Lentiviral modification of mesenchymal stem cells with Notch ligands increases their ability to support hematopoietic stem cell expansion in vitro

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**Introduction:** The probability of successful engraftment and safety of gene-therapy protocols could be greatly increased if gene-modified hematopoietic stem cells (HSC) could be expanded and screened for integration sites prior to transplantation. Also such method would be indispensable for the development of niche models for cell interaction study and *in vitro* drug testing. This study aimed to establish a MSC-based model of HSC microenvironment and to investigate how Notch ligands expression in stromal cells regulates the HSC expansion in a coculture system.

**Methods:** Mesenchymal stem cells from umbilical cord Wharton’s jelly (UC-MSC) were isolated and transduced with lentiviral particles carrying DLL1 or JAG1 genes. To measure transduction efficiency and monitor ligand expression we created bicistronic lentiviral vectors with genes of interest and eGFP. CD34+ cells from cord blood mononuclear fraction were isolated using magnetic bead sorting and cultured with Notch ligand expressing MSC. After 2 weeks phenotype of cells in suspension was analyzed using FACS.

**Results:** Immunophenotypic analysis has shown that after 2 weeks of HSC cultivation, UC-MSC with expression of DLL1 provided 5.7-fold (±1.73) increase of CD34+ cell numbers in suspension fraction compared to cytokine alone conditions and 2.8-fold (±1.23) compared to control UC-MSC. DLL1-MSC was the cell type with ability to support CD34+CD38- population which is considered more undifferentiated than CD34+CD38+. Cultivation of CD34+ cord blood HSC with JAG1-expressing MSC and in cell-free conditions led to 0.9- and 0.6-fold decrease of total CD34+ cell population.

**Conclusion:** Our data indicates that coculture of HSC and DLL1-expressing UC-MSC led to expansion of undifferentiated CD34+ fraction of hematopoietic stem cells. Thus DLL1-MSC can be used as a supportive layer for HSC expansion experiments and provide better reproducibility and lesser batch to batch variation than bone marrow MSCs. This study is ongoing. Currently, we are modifying lentiviral vectors with antibiotic resistance genes which will allow establishment of cell lines with more stable expression of Notch ligands.

**SW04.S21–35**

Human adipose-derived stem cells chondrogenic potential in 3D GAlPAA scaffolds developed for cartilage regeneration

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Cartilage tissue engineering currently requires novel strategies for tissue functional regeneration in cartilage defects. The aim of this study was to comparatively investigate human adipose derived stem cells (hADSCs) chondrogenic potential when embedded in novel gelatin-alginate-polyacrylamide (GAlPAA) 3D scaffolds
designed for cartilage tissue reconstruction versus the control gelatin-alginate (GAI) scaffolds. Cells were seeded on the surface of GAI and GAIPAA and they were allowed to populate deeper layers of the matrices until 3D culture was achieved. These 3D systems were then exposed to a chondrogenic induction cocktail for 28 days and the differentiation process was monitored at 7, 14 and 28 days. Chondrogenic markers SRY (sex determining region Y)-box 9 (Sox9) and Chondrocyte expressed protein 68 (CEP68) gene expression was assessed via qPCR, while the protein levels of the same markers were qualitatively evaluated by confocal microscopy. Cartilage oligomeric matrix protein (COMP) protein expression was assessed using ELISA, whereas scaffold structure was analyzed by SEM studies. Cytoskeleton changes during the chondrogenic differentiation process and cell attachment to the material (actin, tubulin) were visualized by confocal microscopy.

hADSCs displayed chondrogenic potential of differentiation. Sox9 proved to be an earlier inducer of chondrogenesis, displaying high levels of gene and protein expression at the beginning of the process. Extracellular matrix markers CEP68 and COMP displayed an upregulated profile at 14 and 28 days post chondrogenic induction, as compared to the initiation stages of the process.

The upregulated chondrogenic pattern revealed by these markers suggests that GAIPAA could be an appropriate 3D bioconstruct for the regeneration of cartilage defects, considering its good biocompatibility and ability to support in vitro hADSCs chondrogenic differentiation.

**SW04.S21–36 Regulation of stem cell differentiation and DNA damage responses by p53**

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Functional p53 is required for maintaining the genomic integrity of stem cells following genetic insult. Stem cells respond to DNA damage by inducing arrest, undergoing differentiation, or mediating apoptosis. Failure to respond correctly by eliminating or repairing the damaged cells promotes the accumulation of oncogenic mutations and begins the progress towards cancer. We have examined the role of p53 in controlling embryonic stem (ES) cell responses to DNA damage, p53 activation and differentiation induction. A combination of flow cytometry, Western blotting and gene expression profiling were utilised to assess functional changes during differentiation and recovery from damage. ES cell lines varying in p53 status were examined along with cells containing various missense mutants. The absence of functional p53 prevented ES cells from undergoing apoptosis or cell arrest following DNA damage. However, the expression of differentiation markers was enhanced despite maintenance of a stem cell phenotype. In contrast, cells retaining wild-type p53 respond to DNA damage by undergoing apoptosis, differentiating and losing markers of stemness. Furthermore, when mutant p53 was present, specific differentiation programs were instigated. These insights provide clarification of the complex roles p53 has in controlling ES cell fate.
Human embryonic stem cell (hESC) lines represent a cell population that has unlimited replicative capacity and can differentiate into endoderm, mesoderm, ectoderm and germ line. The presence of animal products in human embryonic stem cell culture medium and feeder cell populations drives two main concerns. First, animal products may contain toxins or immunogens that evoke an immune response and use of these products increases the risk of hESC contamination by animal pathogens. Second, feeder cells present such complication as cellular contamination. To solve these problems, several feeder free systems for hESC culturing were created. In this study we analyses karyotypes of established cell lines (eMCS) lines suggest stable, not reconstructed karyotype. Nevertheless, there are no definite answers about eMCS karyotypic stability after in vitro cultivation. According to one data in vitro cultivation leads to karyotypic rearrangements. Another data show that MSC sustain karyotypic stability during long-term cultivation. Fundamental research in this area should be done needed to assess the possibility of using eMCS cultures therapeutic purposes.

In this study we analyses karyotypes of established cell lines of eMCS derived from desquamated (shedding) endometrium in menstrual blood on different passages. This cell lines were maintained on DMEM/F12 medium with 10% of fetal bovine serum, 1% antibiotic antimyotic mixture and 1% glutamine. Metaphase chromosomes were banded using trypsin followed by Giemsa. Multipotency of these established eMSC was confirmed by their ability to differentiate into other mesodermal cell types, such as osteocytes and adipocytes. Besides, the isolated eMSCs partially (over 50%) express the pluripotency marker SSEA-4, but do not express Oct-4.

On early passages (3–6 passage) in these eMSC lines most of the cells display karyotypic stability but some cells with karyotypic abnormalities were found. Revealed abnormalities were chromosomes number variety, ectopic conjugation between non homologues chromosomes, isochromosomes, and chromosome beaks. However, this genomic destabilization has accidental origin and can be a result of stress caused by initiation of cultivation in vitro.

Karyotypic analysis on 13–15 passages shows that after cultivation the cell population in most consists of cells with normal diploid karyotype. Cells with karyotypic instability also were found, but less than on 3–6 passage.

Due to the fact that all cell lines have limited lifespan and undergo replicative senescence we believe that this karyotypic instability do not lead to spontaneous transformation that provides immortalization.
The combination of GSK3 and MEK1,2 inhibitors has been known to promote self-renewal of mouse embryonic stem cells (mESCs), however, the underlying mechanisms remain elusive. We have shown that MEK1,2 inhibition in mESCs results in accumulation of beta-catenin protein but not mRNA. MEK1,2 inhibitor PD0325901 increases E-cadherin to beta-catenin binding, which can underlie the regular compact colony morphology observed upon MEK1,2 inhibition. MEK inhibition alters the expression of some self-renewal/differentiation-related genes, inhibiting beta-catenin-dependent transcription of early mesoderm marker T/Brachyury and enhancing Nanog mRNA expression, and thus shifting the effects of GSK3 inhibition to facilitate pluripotency maintenance.

Both GSK3 and MEK1,2 downstream target ERK1,2 have been shown to regulate the activity of mTOR (mammalian target of rapamycin), the kinase which regulates protein synthesis, cell metabolism, autophagy, and which activities have also been implicated in the regulation of stem cell state. Activity of both mTOR-containing complexes is relatively low in undifferentiated mESCs and increases upon LIF withdrawal and differentiation. Interestingly, both GSK3 and MEK1,2 inhibition attenuate mTOR-dependent phosphorylation of 4E BP1 and Akt, as well as downstream mTOR target S6 ribosomal protein. Inhibition of mTOR activity with rapamycin and PP242 in undifferentiated mESCs decreases cell population growth rates and slightly decreases clonal viability, but does not alter cell cycle distribution of the cells. The expression of differentiation markers (T, Fgf5) is not induced even upon prolonged treatment of mESCs with mTOR inhibitors. However, rapamycin and PP242 do not interfere with these markers expression during early stages of differentiation, but by day 4 after LIF withdrawal mESCs grown in the presence of mTOR inhibitors undergo massive apoptosis. Taken together, our results suggest the convergence of Wnt and MEK/ERK pathways on the few levels to promote self-renewal and pluripotency of mouse embryonic stem cells.

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SW04.S21–42
Effect of mesenchymal cell delivered TRAIL and/or Dkk-1 on cancer cell viability
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Objective: To investigate the anti-cancer effect of mesenchymal cell delivered tumor necrosis factors related apoptosis inducing ligand (TRAIL) Dickkopf-related-1 (Dkk-1), we have transfected human tooth germ (hHTG) derived MSCs, with TRAIL and Dkk-1.

Methods: The conditioned medium (CM) of transfected hTGSCs was collected when the cells reached to 70% confluency. Effects of this CM on human breast cancer cell line MCF-7 and human metastatic neuroblastoma cell line SH-SY5Y were measured using MTS assay and real time PCR analysis of apoptotic markers caspase3 and p53. Results were compared with cells treated doxorubicin and paclitaxel.

Results: The results showed CM of non-transfected hTGSCs increased the survival of MCF-7 and SH-SY5Y cells treated with doxorubicin and paclitaxel. Genetically modified MSCs secreting TRAIL led to a 45% decrease while MSCs secreting Dkk-1 resulted in 27% decrease in the cell viability, which was comparable to doxorubicin and paclitaxel treatment. The double transfection of TRAIL-Dpp-1, on the other hand, led to a 65% decrease in the survival of MCF-7 and SH-SY5Y cells. Treatment of cancer cell lines with CM from TRAIL and/or Dpp-1 MSCs demonstrated approximately a twofold decrease in Bcl-2 and Stat3 (proliferation genes), while increasing the expression of the expression of Bax, FADD, and caspase3.

Conclusion: Our findings demonstrated that MSCs play roles in growth of tumor cells by decreasing the effect of anti-cancer drugs on cancer cells. On the other hand, genetically modified HTG derived MSCs expressing TRAIL and Dkk-1 might be a potential gene and/or cellular gene therapy approach to treat certain types of cancer.
**SW05 Biochemical Mechanisms of Immune Defense**

**SW05.S22 Molecular Basis of Autoimmunity (V-S22)**

**SW05.S22-1 Tumor necrosis factor alpha trigger Caspase-3 gene expression in platelets**

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Tumor necrosis factor alpha (TNF-α) is a potent pro-inflammatory cytokine which is responsible for diverse range of signaling within cells, can induce cellular proliferation, survival, differentiation or necrotic/apoptotic cell death. Platelets are circulating blood cells that do interact with the many molecules and respond to different stimulants. During inflammation TNF-α increased in circulation especially how to act on platelets is not yet fully been mined. TNF-α binding on platelets surface TNF-R1 and TNF-R2 receptors were measured with flowcytometry. Platelets were incubated with TNF-α (100 pg/ml) and/or WP9QY (1 μM, TNF-α Antagonist) in activated with ADP (5 μM). Caspase-3 protein expression were determined by western blotting using β-actin as a control and Caspase-3 gene expression were determined by qRT-PCR. Caspase-3 activity by ELISA was measured in supernatant obtained. TNF-α binding on TNF-R1 (48.63%±12.23) and TNF-R2 (6.18%±3.15) were found to have receptors to both on the platelets. Caspase-3 gene expression levels increased significantly in the TNF-α group compared with control group and decreased in WP9QY compared TNF-α group (p < 0.001). Caspase-3 protein expression was similar to all group in caspase-3 gene expression and pro-caspase-3 bands were detected with western blotting in the samples (p < 0.001- p < 0.01). Caspase-3 activity were significantly higher in TNF-α group than in WP9QY groups respectively (p < 0.01). In conclusion, TNF-α binds to two TNF receptors, TNF-R1 and TNF-R2 on platelets surface. TNF-α can induce apoptosis cascade by activating caspase-3 and the convergence of the extrinsic and intrinsic pathways occurs at the proteolytic activation of caspase-3. This study may be shed light on the mechanisms of inflammation induces hemorrhage in thrombocytopenia.

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**SW05.S22-2 Vitamin D receptor gene polymorphism FokI in rheumatoid arthritis and associated osteoporosis**


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Vitamin D deficiency is linked to increased susceptibility to the development of rheumatoid arthritis (RA) and is found to be associated with disease activity in patients with RA. Vitamin D receptor gene (VDR gene) polymorphism FokI is functionally active. It is characterized by the substitution of thymine (T) by cytosine (C) in the start codone of the exon 2. Presence of FokI restriction site (f) results in the synthesis of 427 amino acids long protein, while the absence of restriction site results in 424 amino acids long protein. Shorter protein has a higher transcriptional activity and stronger affinity for the active form of vitamin D.

The aim of this study was to examine the association of FokI VDR gene polymorphism in patients with RA and in RA patients with associated osteoporosis.

The FokI polymorphism was determined in 67 patients with RA (18 of them with associated osteoporosis, OP) and 72 healthy controls using PCR-RFLP method.

Obtained results show significantly higher prevalence of Ff+fversusFF genotype in patients with RA (χ² = 10.77, p = 0.001), as well as in RA patients with associated OP (χ² = 13.38, p < 0.001) compared to controls. No differences in genotype frequencies between RA and patients with associated OP were observed. Allele f frequency is significantly higher in patients with RA (χ² = 10.64, p = 0.001) and in patients with associated OP (χ² = 10.61, p = 0.001) compared to controls.

Our results indicate that the presence of VDR FokI polymorphism is associated with RA and RA with associated osteoporosis susceptibility.

**SW05.S22-3 Investigation of kappa B alpha inhibitor promoter polymorphisms in patients with primary Sjogren Syndrome**

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Primary Sjogren’s syndrome (pSS) is an autoimmune disease that is common all over the world and in our country. The underlying genetic basis of the disease is influenced by the environmental factors. NFxB is a transcription factor associated with immune response, antiapoptotic genes and proinflammatory cytokines. IκBα binds to NFκB in cytoplasm and regulates the transcriptional activity by interacting with transcription factors. Polymorphisms in the promoter region of IκBα is thought to play a role in the pathogenesis of inflammatory and autoimmune diseases. Several polymorphisms were identified in the promoter region of the IκBα gene. To our knowledge there is only one study investigating IκBα promoter region-881 A/G and –826 C/T polymorphisms in Primary Sjogren’s syndrome. This study, IκBα promoter region –881 A/G and –826 C/T polymorphisms and investigated the relationship between pSS. Hundred and three patients diagnosed with primary Sjogren’s syndrome, and 99 healthy subjects were included to the study. IκBα promoter region –881 A/G and –826 C/T genotypes were determined by PCR and RFLP methods. In our study, patient and the healthy control groups –826 C/T and –881 A/G polymorphism frequencies did not differ significantly. As a result, our study showed no association between IκBα promoter region polymorphisms and pSS.
Ets-2 protein is a transcriptional repressor of the HIV-1 virus

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The HIV1 virus is transcriptionally active in activated CD4 T-cells, and inactive in naive CD4 T-cells. Our previous work has shown that Ets-2 is a candidate transcriptional repressor of HIV1-LTR in naive CD4 T-cells, because the −279 to −250 upstream region of HIV1-LTR (RATS, Repressor Activator Target Sequence), that participates in HIV1-LTR transcriptional silencing, enhances the AAGGAG Ets-2 binding site. In the present proof of concept study, we investigated whether Ets-2 represses the expression of HIV1.

We measured the levels of Ets-2 mRNA in peripheral blood-derived T-cells and 11 leukemic cell lines, before and after activation of the cells with PMA/Ionomycin (P/I). Ets-2 mRNA was synthesized in T-cells and in the most cell lines when cultured in plain culture medium (CM), but its synthesis was severely reduced when the cells were stimulated. We transfected cells with (i) the plasmids HIV1-LTR-CAT, 2xRATS-CAT (two copies of RATS sequence), 2xmutantRATS-CAT (carries a point mutation in the Ets-2 binding site) and CMV-CAT (control), (ii) pCDNA3-ets2 (for ets-2 overexpression), (iii) ets-2 shRNA (to silence ets-2 expression in the cells). When T-cells were transfected with HIV1-LTR-CAT, 2xRATS-CAT and CMV-CAT, in CM there was no transcriptional activity of the genes. After activation of the cells, the expression of the transfected genes increased, except CMV-CAT. Co-transfection experiments in Jurkat cells with increasing amounts of pCDNA3-ets2, led to a gradual reduction of HIV1-LTR-CAT and 2xRATS-CAT transcriptional activity, upon cell stimulation with P/I, but not of CMV-CAT. No transcriptional response was observed for the 2xmutantRATS-CAT reporter gene. Cotransfection experiments with HIV1-LTR-CAT, 2xRATS-CAT and ets-2 shRNA led to an increase in both reporter genes activity but not for 2xmutantRATS-CAT and CMV-CAT.

Our results confirm the role of Ets-2 as a transcriptional repressor of HIV1. This HIV-LTR-RATS-mediated repression may account for the low-level transcription and replication of HIV1 in naive T-cells, and contribute to the viral latency and maintenance of viral reservoirs in patients, despite long-term therapy.

The role of endogenous glucocorticoids in glucose metabolism and immune status of MIF-deficient mice

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Macrophage migration inhibitory factor (MIF) is a pluripotent cytokine involved in promotion of inflammation, regulation of glucose metabolism and inhibition of glucocorticoid (GC) action. It is already established that MIF-deficient (MIF-KO) mice develop glucose intolerance and obesity and that their immune status is skewed toward anti-inflammatory. Since MIF is a counter-regulator of GC action and elevated GC are implicated in diabetes development, we hypothesized that MIF absence enables elevation of GC which in turn cause the observed condition. Our results confirm that MIF-KO mice had elevated levels of circulating corticosterone measured by ELISA compared to their wild type (WT) counterparts C57BL/6 mice. However, lower expression of glucocorticoid receptor (GR) was detected in pancreatic islets by real time PCR (RT-PCR) and Western blot (WB) analysis. Similarly, liver and adipose tissue of MIF-KO mice possessed lower content of GR to the one observed in WT mice. In contrast, flow cytometry analysis showed a significant up-regulation of GR expression in MIF-deficient lymph node cells. Treatment with a GR inhibitor RU486 improved tolerance to glucose in MIF-KO mice and restored normoglycemia. Although RU486 treatment did not alter the level of glucose receptor GLUT2 in pancreatic islets and liver (WB), it enhanced insulin secretion and up-regulated insulin-triggered Akt phosphorylation within hepatic tissue (ELISA and WB, respectively). Finally, RU486 changed lymphocyte secretion pattern in MIF-KO mice by increasing levels of IFN-gama and TNF-alpha, and decreasing TGF-beta and IL-4 secretion (ELISA). Our results indicate that dysregulated glucocorticoid secretion and GR expression in the absence of MIF possibly contributes to the development of glucose intolerance and immunosuppression in MIF-KO mice.

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Release of ADAM15 exosomes and its functional characterization

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A disintegrin and metalloproteinase 15 (ADAM15) is a type-I transmembrane glycoprotein having an Arg-Gly-Asp (RGD) motif in its disintegrin-like domain. Present study demonstrates that ADAM15 is constitutively released via exosomes, and the ADAM15 exosomes exert tumor-inhibitory functions. The extra-cellular release of ADAM15 is stimulated by phorbol 12-myristate 13-acetate, a typical protein kinase C activator, in several tumor cell types with corresponding decrease in plasma membrane-associated ADAM15. Exosomes rich in ADAM15 display enhanced binding affinity for integrin v8f3 in RGD-dependent manner and inhibit vitronectin- or fibronectin-induced cell adhesion, growth and migration as well as in vivo tumor growth. Further experimental investigation revealed that ADAM15 exosomes are actively released from human macrophages and the release of exosomal ADAM15 is significantly increased by lipopolysaccharide stimulation, suggesting that the ADAM15 release is closely associated with immune cell differentiation and activation. The tumor-suppressive ADAM15 exosomes effectively extend the survival of tumor-bearing mice. This work suggests a fundamental role of ADAM15 for exosome-mediated tumor suppression as well as biological significance of ADAM exosomes in anti-tumor immunity.

Study of catalytic antibodies and their implication in autoimmune disease

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The study of catalytic antibodies or abzymes has come a long way since the realization that the variable regions of antibodies could potentially resemble the active sites of enzymes and hence be catalytically active [1]. In the late 1980s and the early 1990s, many ab-
zymes were observed on the background of autoimmune disease [2]. This pointed to the existence of a link between autoimmunity and the formation of natural catalytic antibodies. More recently, enzymes have also been discovered in normal physiological states [3]. Despite the outstanding advances in the study of catalytic antibodies, there are many questions yet to be answered about the nature of these molecules and their role in the immune system. To address these questions, a statistical analysis was performed on the genetic sequences of 40 abzymes. It was shown that these antibodies display a high conservation degree with their germline counterparts, however the low rate of mutations induces a significant modification of physico-chemical properties of the amino acids. This suggests that there is a difference between the maturation process of abzymes and binding antibodies. It was also shown that catalytic antibodies are more frequently expressed by rare gene subgroups, possibly explaining their high occurrence in autoimmune disease [4]. In order to confirm these findings, we are conducting an experimental study comparing the elicitation of catalytic antibodies in four different immune repertoires, which are represented by phage displayed combinatorial libraries of single chain antibody fragments (scFv). Four individually tagged phagemid vectors are constructed as means for a simplified selection process. The elicited catalytic antibodies are selected by using a suicide substrate as a trapping agent. Here we focus on the analysis of the gene subgroups, catalytic residues, and somatic mutations responsible for the catalytic function of these antibodies. These results will lead to a better understanding of the characteristics of catalytic antibodies and their link to autoimmune disease.

References

SW05.S22–9
Osteopontin, IL-10 and IL-12 in active and remission stages Behçet’s disease
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Behçet’s disease (BD) is a multisystem disease with unknown etiology, characterized by aphthae, genital ulcers and uveitis. It has been suggested that many cytokines involved in BD pathogenesis may play an important role in these inflammatory responses. Osteopontin (OPN) has been shown to promote Th1 type immune response and thus has been implicated in the pathogenesis of autoimmune disease. OPN is generally classified as a proinflammatory cytokine, because it stimulates pro-inflammatory cytokine IL-12 and suppresses anti-inflammatory cytokine IL-10.

The aim of this study was to investigate the role of OPN in both active and remission stages of Behçet’s disease. This study was conducted on 22 patients with Behçet’s disease, 18 patients with systemic lupus erythematosus (SLE) and 18 healthy controls. The patients with BD were categorized according to the clinically active and remission stages. Serum levels of OPN, IL-10 and IL-12 were measured by enzyme linked immunosorbent assay.

OPN levels in active BD and SLE patients were found higher than healthy controls (p < 0.05 and p < 0.01, respectively) but showed no significant difference between active and remission stages of BD. In SLE patients, OPN levels were positively correlated with both CRP and erythrocyte sedimentation rate (ESR) values (p < 0.05 and p < 0.05, respectively). IL-10 and IL-12 levels in SLE patients were significantly higher when compared with both active BD patients (p < 0.01 and p < 0.001) and healthy controls (p < 0.001, p < 0.001, respectively).

Our findings suggest that OPN is possibly involved in pathogenesis of active BD. However no significant differences were noted between active and remission stages of BD patients. These findings need to be confirmed by larger prospective studies.

SW05.S22–9
Scleroderma sera induce reactive oxygen species (ROS)-dependent activation of collagen synthesis in human pulmonary vascular smooth muscle cells*

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Background: Vascular disease is a dominant component of Systemic Sclerosis (SSc) pathogenesis. Hypoxia and oxidative stress have been linked to endothelial injury, intimal hyperplasia and progressive vessel occlusion in SSc, and the aberrant function of Vascular Smooth Muscle Cells (VSMCs) has been associated with the initiation and the amplification of this process.

Objectives: We hypothesize that in SSc patients circulating factors may contribute to vascular damage by exerting pro-oxidant and pro-fibrotic effects involving VSMC. METHODS: The generation of reactive oxygen species (ROS) and the activation of collagen synthesis has been investigated in primary Human Pulmonary Vascular Smooth Muscle Cells (HPVSMC) after exposure to sera obtained from 15 SSc patients with pulmonary arterial hypertension (PAH) and 15 age/sex matched healthy donors (HD). Intracellular ROS levels were assessed using the general oxidative stress indicator dichlorodihydrofluorescein diacetate (H2-DCFDA). Collagen type I (CTI) promoter activity was investigated by using a GFP-based lentiviral vector driven by the human collagen type I promoter, while collagen protein expression was assessed by ELISA. In selected experiments pretreatment of cells with diphenyl iodonium (DPI), a general flavoprotein inhibitor, was performed.

Results: HPVSMC treated with SSc sera significantly increased intracellular ROS levels (338 ± 84 RFU) compared to HD sera (117 ± 24 RFU). DPI effectively counteracted the raise of intracellular ROS, implicating the involvement of Flavin Oxidases (FO) in this process. Interestingly, the pro-oxidant stimulus provided by SSc sera was associated to a parallel increase of both CTI promoter activity and collagen protein expression in HPVSMC (360 ± 87 RFU, SSc versus 28 ± 8 RFU, HD). Also this effect was abrogated by pretreatment with DPI suggesting that FO-derived ROS production can drive pro-fibrotic responses in HPVSMC.

Conclusion: This study support the existence in SSc patients affected by pulmonary vascular disease of circulating factors driving oxidative stress and collagen synthesis in human VSMC. While such potential mediators of pathological vascular remodeling remain to be identified, our results further support the consideration of antioxidant-based therapies in the treatment of SSc vascular disease.

RFU = Relative Fluorescence Units.
Innate immunity is characterized by its ability to recognize a wide range of pathogens through a limited number of receptors, mainly TLRs, and the recent NLRs, which consist of soluble proteins that survey the cytoplasm for ‘danger signals’ that advertise the presence of intracellular invaders, forming the inflammasomes. Inflammasomes are molecular complexes that activate inflammatory caspases, which are involved in the maturation of cytokines of the IL-1 family. On the basis of the recent evidences demonstrating the important role of the endocannabinoid system in the modulation of several immune responses, we investigated whether endocannabinoids may interfere with inflammasomes activation. In particular, we found that endocannabinoid N-arachidonoylethanolamine and 2-arachidonoylglycerol modulate NLP3-inflammasome dependent caspases-1 expression and production of IL-1β, and IL-18 from human macrophages. Overall, our findings account for a new homeostatic role of the endocannabinoid system in the fine-tuning of those feedback loops, which are crucial for either initiation or resolution phases of inflammation, thus hinting at novel therapeutic opportunities for the treatment of several inflammatory diseases.

**SW05.S22–11**

**Apolipoprotein E gene polymorphisms in chronic periodontitis**


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**Objective:** Periodontal diseases can be related to common systemic conditions, and, as recently described, alterations in blood lipid levels. The aim of this study was to analyze two apolipoprotein E (ApoE) gene polymorphisms in patients with chronic periodontitis (CP) in relation to periodontopathic bacteria and lipid levels.

**Methods:** Four hundred and sixty-nine unrelated subjects were included in this case-control study. Genomic DNA of 294 patients with CP and 175 healthy/non-periodontitis controls were genotyped, using the real-time polymerase chain reaction (RT-PCR) method monitored by SYBR Green, for ApoE (rs429358 and rs7412) gene polymorphisms. Subgingival bacterial colonization was investigated by the DNA-microarray based on a periodontal pathogen detection kit and lipid levels were measured in a subgroup of subjects.

**Results:** There was no evidence for a significant association between ApoE gene polymorphisms and CP (e2/e2 versus e3/e3: OR = 0.63, 95%CI = 0.12–3.15; e2/e3 versus e3/e3: OR = 1.61, 95%CI = 0.84–3.11; e2/e4 versus e3/e3: OR = 1.25, 95%CI = 0.23–6.95; e3/e4 versus e3/e3: OR = 1.11, 95%CI = 0.65–1.88; e4/e4 versus e3/e3: OR = 0.63, 95%CI = 0.15–2.53; allele e2 versus allele e3: OR = 1.26, 95%CI = 0.74–2.14; allele e4 versus allele e3: OR = 0.97, 95%CI = 0.63–1.51). Patients with CP had increased total cholesterol and LDL levels compared to controls (p < 0.05), however no significant differences were found for triglyceride and HDL levels. Gene variability in the ApoE gene influenced LDL levels marginally (p = 0.07) but did not modify total cholesterol, triglyceride and HDL levels or the occurrence of periodontal pathogen in subgingival pockets.

**Conclusions:** In Czech population studied, ApoE genetic variations rs429358 and rs7412 were not associated with susceptibility to and severity of chronic periodontitis or with the presence of periodontopathic bacteria.

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**SW05.S22–10**

**Novel modulators of inflammasomes: insights from the endocannabinoid system**

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Innate immunity is characterized by its ability to recognize a wide range of pathogens through a limited number of receptors, mainly TLRs, and the recent NLRs, which consist of soluble proteins that survey the cytoplasm for ‘danger signals’ that advertise the presence of intracellular invaders, forming the inflammasomes. Inflammasomes are molecular complexes that activate inflammatory caspases, which are involved in the maturation of cytokines of the IL-1 family. On the basis of the recent evidences demonstrating the important role of the endocannabinoid system in the modulation of several immune responses, we investigated whether endocannabinoids may interfere with inflammasomes activation. In particular, we found that endocannabinoid N-arachidonoylthanolamine and 2-arachidonoylglycerol modulate NLP3-inflammasome dependent caspases-1 expression and production of IL-1β, and IL-18 from human macrophages. Overall, our findings account for a new homeostatic role of the endocannabinoid system in the fine-tuning of those feedback loops, which are crucial for either initiation or resolution phases of inflammation, thus hinting at novel therapeutic opportunities for the treatment of several inflammatory diseases.

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**SW05.S22–12**

**Intrinsic defect in B-lymphoblastoid cell lines from patients with X-linked lymphoproliferative disease type 1**

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X-linked lymphoproliferative disease type 1 (XLP1) is associated with mutations in the gene encoding the adaptor molecule SH2D1A/SAP, which is expressed in NK, NKT, T cells and on the narrow stage of B lymphocyte differentiation. SH2D1A binds to cytoplasmic domains of CD150, CD84, CD229, CD244 and CD352 receptors expressed on hematopoietic cells that deliver activation signals following interactions with their cognate ligands. Mutations in SH2D1A are believed to compromise the signaling pathways elicited by these receptors. Few evidence exist that impaired humoral immunity in XLP1 is associated with defective T-cell help. We addressed the question whether B-lymphoblastoid cell lines (B-LCL) from XLP1 patients have intrinsic defect in cell signaling that affect cell activation and proliferation. XLP1 B-LCLs IARC739, 8003, 8005, RP-EBV and Denman were studied in comparison with normal B-LCLs MPI (SH2D1A positive), T5-1 and RPMI-1788 (SH2D1A negative). Despite similar cell surface phenotype, XLP B-LCLs had diminished activation and proliferation in respond to ligation of the B cell receptor (BCR) and CD40, as well as in combination with CD150. These receptors are linked to Akt/PKB and MAPK (ERK1/2, p38MAPK and JNK1/2) signal transduction pathways regulating transcription factor network that control B cell proliferation, differentiation and apoptosis. In XLP1 cell lines CD150-mediated Akt and MAPK activation was not altered, however, BCR-mediated ERK1/2 activation and IkB phosphorylation was significantly reduced. Moreover, XLP1 B-LCLs differ from normal B-lymphoblastoid cell lines by modulation of CD95-mediated apoptosis via CD40 and CD150. In XLP B-LCLs CD150 crosslinking did not affect CD95-mediated apoptosis, unlike in normal B-LCLs. CD40 ligation was synergistic with CD95 in XLP B-LCLs, however, culling of these receptors rescued normal B-LCLs from apoptosis. Our study provides a new evidence for intrinsic defect of B-lymphoblastoid cell lines from patients with X-linked lymphoproliferative disease type 1.
Cigarette smoke (CS), a major source for oxidative stress in the lungs, is the main cause of chronic obstructive pulmonary disease (COPD). Patients suffering from COPD are more susceptible to viral infections resulting in acute exacerbations. Viral infections induce expression of immunoproteasomes (IP) via IFN-gamma signaling on the site of infection. This specialized form of proteasome is destined to improve antigen presentation in infected cells and to efficiently degrade oxidatively damaged proteins. Additionally, IPs protect the organism from autoreactive cytolytic T-lymphocytes, since certain self-peptides are only presented to the immune system during ongoing inflammation. The role of IPs in COPD pathology is unknown.

Expression levels of IP-subunits LMP2 and LMP7 were evaluated in wild type (wt) as well as LMP2/−/− and LMP7/−/− deficient mice in whole lung homogenates. Of note, we observed pronounced expression of IP-subunits in wt lungs compared to other organs. Immunohistochemical analysis of wt lung sections revealed low overall LMP2 expression in alveolar lung cells. To study regulation of IP in vitro, we analyzed expression of IP in different lung cell lines and primary cells. IFN-gamma induced pronounced expression of IP in both, lung epithelial and fibroblast cells, as detected by qRT-PCR and western blotting. We next investigated regulation of IP by cigarette smoke. A549 human lung epithelial cells were treated with extracts of CS for 24 hours up to 3 days, and expression of IP was investigated on RNA and protein level. Long-term treatment of cells with CS-extract resulted in downregulation of basal IP expression. We also investigated the effect on IFN-gamma mediated induction of IP and observed reduced expression of IP subunits in the presence of CS extract. We are currently analyzing IP expression in diseased lungs.

Lowered expression of IPs due to smoke exposure may affect antigen processing of virus and host proteins and thus add on severity and delayed resolution of viral infections in exacerbations.

Multiple sclerosis (MS) is an autoimmune and demyelinating disease of the central nervous system (CNS). It is considered that the formation of MS chronic process in the CNS is determined by excessive production of pro-inflammatory cytokines (IFNγ, TNFα), which are accompanied by a decrease of anti-inflammatory cytokines (IL-10, IL-6). In addition to autoimmune mechanisms glutamate excitotoxicity might contribute to the lesion of neurons in the brain in MS. Glutamate is the major excitatory neurotransmitter in the CNS and is released in large quantities by activated immune cells during MS inflammation. One of the types of glutamate receptors are NMDA-receptors. Now it is proved that the NMDA-receptors expressed on the surface of T lymphocytes and are involved in the regulation of key functions, including the secretion of cytokines, the polarization of subpopulations of T helper cells and the maintenance of calcium homeostasis.

In the present study we investigated the effects of NMDA-receptors blockade on production of pro-and anti-inflammatory cytokines in T lymphocytes of MS patients. Incubation of T cells in the presence of NMDA-receptor antagonist did not alter the expression of mRNA for TNFα and IFNγ, but increased the level of mRNA for CSIF, IFNβ by 25% (p<0.05). In case of TCR-mediated stimulation of T lymphocytes the NMDA-receptor blockade reduced the IL-10 production of nearly two times, TNFα and IFNγ release ~ by 1.5 times, whereas significant changes in IL-6 production were not observed. It was also shown that NMDA-receptors blockade of TCR-activated cells was associated with decreased mRNA content for IFNβ2 and CSIF by 25% (p<0.05), IFNγ by 27% (p<0.05), TNFα by 10% (p<0.05).

Our findings demonstrate that NMDA-receptors of T cells modulate the cytokine production, suggesting that this type of glutamate receptors is involved in the mechanisms of inflammation in MS.

This work was supported by RFBR-Volga Region Grant No. 11-04-97093.
**SW05.S22–16**

**Variants of the IL-23R and STAT3 gene are not associated with Hashimoto’s thyroiditis**


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Interleukin-23 receptor (IL-23R) and signal transducer and activator of transcription 3 (STAT3) polymorphisms are common risk factors for a number of T helper (Th) 17-mediated autoimmune diseases. However, the importance of genetic variations in Th17 pathways to thyroid autoimmunity, and particularly Hashimoto’s thyroiditis (HT), is not fully understood. In this study, we genotyped three single nucleotide polymorphisms (SNPs) within the IL-23R (rs11209026/p.Arg381Gln, rs7530511) and STAT3 (rs744166) genes in 217 Croatian patients with HT and 161 healthy controls using fluorescence resonance energy transfer technology and melting curve analysis of polymerase chain reaction products. None of the tested SNPs or IL-23R haplotypes were associated with HT susceptibility or disease severity. A recent study suggests that the studied IL-23R/STAT3 polymorphisms affecting Th17 signaling efficiency are not major determinants of HT risk in the Croatian population. Further work is necessary to determine if these loci contribute modestly or conditionally to the risk of HT.

**SW05.S22–17**

**The effect of allergic rhinitis treatment on serum ischemia modified albumin levels in children with allergic rhinitis**

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**Background:** In recent years, in parallel with the use of food additives, allergic rhinitis is a major health problem in developed countries. Strategies for the prevention of complications of allergic rhinitis due to hypoxia are required. Ischemia modified albumin (IMA) was considered to be related with increased oxidative stress and as a potential cardiovascular risk factor in recent years. So we aimed to determine the effect of allergic rhinitis treatment on IMA in children with allergic rhinitis.

**Methods:** This study was performed in 40 children (mean age 9.6, min: 5 – max: 16) with allergic rhinitis and 30 healthy children (mean age 8.9, min 7 – max: 13). The serum samples were included at baseline and after 2 weeks symptomatic treatment of allergic rhinitis. IMA levels were determined with a colorimetric assay based on the reduced binding of cobalt (Co II) to serum albumin which is modified by ischemic/hypoxic conditions or free radicals. The results were expressed as ABSU. The changes in biochemical parameters were compared with either Wilcoxon Signed Rank test.

**Results:** IMA levels (1.16 ± 0.10 ABSU) are increased in children with allergic rhinitis compared to control group (0.61 ± 0.19 ABSU) (p < 0.001). But there was no difference between the pre- and post-treatment IMA levels (1.10 ± 0.18 ABSU) in allergic rhinitis (p > 0.05).

**Conclusion:** Our study indicates that IMA levels were increased in children with allergic rhinitis compared to control group, but the treatment of allergic rhinitis does not effect IMA levels. For this reason, cardiac risks should be follow in these children. In order to go up further in the effects of hypoxia, new studies should be studied in older allergic rhinitis patient.

**SW05.S22–18**

**Polymorphic Prnp-flanking genes, but not Prnp itself, controls phagocytosis of apoptotic cells**

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Prnp−/− mice lack the cellular prion protein PrPc and are resistant to prion infections, yet the molecular function of PrPc remains elusive. Numerous phenotypes reported in Prnp−/− mice were not reproduced in different laboratories, suggesting interference by genetic confounders. In this study we analyzed the role of confounding genes in the inhibition of phagocytosis, a cell-autonomous phenotype previously reported in Prnp−/− mice. Using formal genetics, genomic, and transcriptomic analyses, we provide evidence that the regulation of phagocytosis previously ascribed to PrPc is instead controlled by genes flanking the Prnp locus. Our findings offer an alternative interpretation of previous studies on the function of PrPc as a phagocytosis modulator and illustrate the requirement for stringent genomic approaches to identify confounding flanking genes in studies using gene-targeted mice.

**SW05.S22–19**

**Dioxin-mediated regulation of genes involved in cytokines production by macrophages**


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TCDD is the most toxic among the dioxin xenobiotics and induces a broad spectrum of biological responses, including also immunotoxicity and cancer [1]. Macrophages are key regulators of the innate immune response, as well as one of the first types of cells to respond to stress, so the study of the action of TCDD in these cells is important. TCDD mediates gene expression via AhR/ARNT transcription complex activation, which binds to dioxin responsive elements (DRE) in the regulatory regions of the inducible genes. TCDD acts as a stimulator of some cytokines [2] and our analysis [3] showed that the list of stimulated cytokines is not yet complete. Also the possibility of direct as well as indirect regulation of cytokine synthesis via transcription factors (TFs), through DREs in its promoters was shown.

**Methods and Algorithms:** By using the SITECON software package the regulatory regions of the IL12A, IL12B, IL4, RELA, ATF3 genes were searched for DREs. EMSA, Real-time PCR and ELISA experiments were performed on U937 macrophage-like cells to test functionality of predicted sites.

**Results:** Obtained data demonstrates functional activity of DREs in IL12A, IL12B, IL4, REL and RELA gene promoters via AHR signal pathway. The expression dynamics of mRNA IL12A and IL12B genes also evidence the indirect TCDD-mediated modulation of these genes via TFs, which obtain DREs in its promoters.
Abstracts

**Conclusion:** Activation of these genes by Ahr ligands can lead to change of regulation key cytokines and this may be a potential cancerogenic pathway induced by dioxin.

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**References**

**SW05.S22–20**

Role SNPs of CD40 in predisposition to multiple sclerosis

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The interactions of CD40 and CD40L have been known to critically regulate B-cell responses. Thus, CD40 is a likely candidate to play roles in autoimmune diseases such multiple sclerosis (MS) in which activated T and B cells cause pathology. In 2009, Australia and New Zealand Multiple Sclerosis Genetics Consortium was identified risk-associated SNP upstream 6 kb of CD40 on chromosome 20q13 (rs6074022, p = 1.3 × 10^{-5}). [1]. An association between rs6074022 and MS was also identified in a GWAS based on a cohort from the United Kingdom [2]. However, in the meta-analysis reported in 2011, the association between SNP rs6074022 and MS did not reach the GWAS significance level (p = 4.91 × 10^{-6}) [2]. Latter, Blanco-Kelly F et al. identified association between rs1883832, a polymorphism located at −1 from the start codon of the CD40 gene, and MS [3].

The aim of this work was to study the association between these two and other two CD40 SNPs and multiple sclerosis from Russian Federation. Moreover we would like to estimate location causative SNP.

Determination of genotypes of CD40 SNPs (rs6074022, rs1883832, rs1535045, rs11086996) was performed by Real-time PCR. Statistical analysis was performed using the R-language.

In this work, we studied the association between four CD40 SNPs and multiple sclerosis in 1679 cases and 879 controls from Russian Federation. Two SNPs were associated with MS: rs6074022 (OR = 1.27, 95%CI = (1.12–1.45) p = 0.0003, per allele) and rs1883832 (OR = 1.20, 95%CI = (1.05–1.38) p = 0.007, per allele), whereas rs1535045 and rs11086996 were not.

These two associated SNPs are in strong linkage disequilibrium (D' = 0.82, r2 = 0.59) and mapped within a promoter region of the gene. Maximum likelihood analysis showed that the model, including rs6074022, is significantly better (p = 0.01) than model, including rs6074022 and rs1883832. Summarizing the data, we may speculate that the functional variant(s) is likely to be located in the upstream region of the gene CD40 and is in higher LD with rs6074022 than with rs1883832.

**References**

**SW05.S22–21**

T cell development in mice with increased alternative NF-κB pathway

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NF-κB family members are a group of transcription factors which play major role in development and functioning of immune system. Mutations in NF-κB proteins lead to defects in innate and adaptive immunity, inflammation and cancer. Conventionally three pathways of NF-κB signaling are recognized: canonical, non-canonical (or alternative) and hybrid. The key step of alternative nfkβ pathway is partial proteolysis of p100 precursor protein and formation of p52, which in complex with RelB binds the DNA. Recent studies show that nfkβ signaling is involved in T and B cell development in mice. The use of gene knock-out demonstrates the importance of NF-κB in commitment and maturation of mouse lymphocytes. In the current research we focused on T cell development in thymus of mice with truncated p100, which leads to over activated alternative NF-κB signaling. Previously, we have shown that increased alternative NF-κB signaling leads to defect in B cell development in bone marrow and impaired T cell independent response type 2. Therefore our aim was to investigate the development of T lymphocytes in mouse model of p100 mutation. T cell development starts from bone-marrow derived precursor seeding the thymus, where it commits to T cell lineage, recombines T cell receptor and finally leaves thymus as a self-competent and self-tolerant naïve T cell. Alternative NF-κB pathway has been shown to play role in T cell development. Mice with p100 protein truncation are characterized by increased activity of alternative NF-κB pathway due to constant p52-RelB presence in the nucleus. These mice are characterized by severe loss of double positive (DP) T cell precursors and premature thymic atrophy which complicates its study. We hypothesized that this might be due to high corticosteroid levels. To overcome this p100 mice were crossed to GRdim mice carrying missense mutation in dimerization domain of glucocorticoid receptor. GRdim mutation rescued severe apoptosis of DP precursors. This allowed dissecting p100 mutation input itself and its secondary effects. Analysis of mainstream T, gd T and dendritic cell populations did not reveal any major defects. However iNKT cell population seemed to have enhanced maturation with respect to NK1.1− to NK1.1+ transition. Interestingly this effect was opposite in p100 to wt bone marrow chimeras, namely we observed a mild block in NK1.1+ cell maturation. Competitive bone marrow chimeras revealed general defect in T cell compartment of p100 mice and also of NKT cells. Fetal thymus transplantation of p100 mice to nude mice showed that it sustains normal T cell development but iNKT cell maturation was blocked on later stage. These results indicate that overexpression of RelB leads to cell intrinsic NKT cell defect reflected by late maturation block.
SW05.S23 Immunohistochemistry and Bioengineering (V-S23)

SW05.S23–1
New gene targets of LTbR signaling pathway in mouse lymphoid stroma
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Lymphotixin beta receptor (LTbR) provides critical signal in development of secondary lymphoid organs. We performed Illumina expression profiling assay to identify set of genes which are directly or indirectly regulated by LTbR signaling in spleen stromal cells. Comparison of LTbR-knockout mice with their wild type counterparts revealed that in knockout stroma 505 genes are downregulated and 25 genes are upregulated (fold change is no <1.5). Among them there are known LTbR (Madcam1, Cxcl13, Ccl21, Il-7, Vegfa) and NFkB targets as well as new genes never described as LTbR-dependent. Microarray data was validated by PCR and Northern blot. Several genes were chosen for further analysis. Immunohistochemical analysis was conducted for the protein product of clusterin gene which mRNA level is threefold lower in knockout animals. Clusterin is a multi-functional protein existing in two forms. It participates in tissue remodeling, apoptosis, lipid transport, complement-mediated cell lysis, and serves as an extracellular chaperone. All subsets of spleen and lymph node stromal cells appeared to be immunopositive for clusterin, except for marginal reticular cells. It is present in heterodimeric form as confirmed by Western blot. Precise role of this protein in immune system remains to be investigated.

SW05.S23–2
The immunomodulatory role of endogenous glucocorticoids in ovarian cancer
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Background: Tumour-infiltrating myeloid-derived suppressor cells (MDSC) or tumour-associated macrophages (TAM) which are abundant in ovarian cancer show a high expression of the enzyme 11Beta-Hydroxysteroid dehydrogenase I (11b-HSD1). This enzyme is essential for the conversion of biologically inactive cortisol into active cortisol which has been detected in ascitic fluid and tumour exudates from ovarian cancer patients. Considering that cortisol has strong suppressive effects on all immune cells, we hypothesize that the activation of endogenous glucocorticoids by MDSC or TAM may contribute to the immune escape of ovarian cancer.

Material and methods: Using immunohistochemistry, real-time PCR, luminescent immunoassays (LIA), immunofluorescent double staining and adoptive transfer of glucocorticoid receptor knock out immune cells into immune deficient mouse model for ovarian cancer.

Results: We found that 11b-HSD1 enzyme is highly expressed in human and murine ovarian cancer tissue. Luminescent immunoassays (LIA) showed elevated cortisol levels in serum, ascites and tissue exudates from ovarian cancer patients as compared to healthy controls. Immunofluorescent double staining revealed a co-localization of 11b-HSD1 with CD14, CD68, and CD85, but not with EpCAM. Expression of 11b-HSD1 can thus be attributed to tumour associated macrophages (TAM) or myeloid derived suppressor cells (MDSC). To test our hypothesis about activation of endogenous glucocorticoids by immune cells may contribute to the immune escape of ovarian cancer is now being tested in PTENloxP/loxP, loxP-Stop-loxP-krasG12D mice which spontaneously develop ovarian cancer after intra-bursal injection of adenooviral Cre recombinase. The ongoing experiments involve adoptive transfer of glucocorticoid receptor knock out immune cells as well as pharmacological inhibition of 11b-HSD1 which shall be combined with various immune stimuli. In a first functional in vivo assay, the adoptive transfer of glucocorticoid receptor-deficient T cells led to increased immune cell infiltration of the tumour tissue – which did not translate into prolonged survival. Instead, infiltrating T cells assumed mostly a Foxp3+ (regulatory) phenotype and survival was even shortened.

Conclusion: We thus propose that endogenous glucocorticoids exert immunomodulatory functions in ovarian cancer. Their putative role in tumour immune escape, however, needs to be assessed in context of further tolerogenic mechanisms that may be simultaneously present.

SW05.S23–3
Pancreatic polypeptide in chronic pancreatitis or/and diabetes
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Introduction: Pancreatitis is a disease with increasing incidence which can be divided into an acute and a chronic form. In both acute and chronic pancreatitis (CP), changes in plasma concentration of pancreatic polypeptide (PP) and its regulation have been reported.

Aims and Methods: Therefore, ours studies have investigated tissue expressions of PP in patients with chronic pancreatitis or/and diabetes as a diagnostic maker and as a therapeutic option to treat pancreaticogenic diabetes mellitus. Pancreatic tissue was taken during surgical intervention. The sections were subject to a histopathological examination and in 19 cases chronic pancreatitis was identified. In eight patients was found diabetes. The control pancreatic specimens were obtained from four persons during the collection of organs for transplantation. Immunohistochemical localization of PP in tissue was performed using the LSAB2-HRP visual test with polyclonal PP antibodies. The intensity of immunohistochemical reaction was calculated with the semiquantitative Digital imaging methodology.

Results: Patients with CP showed significantly increase expression of PP in the pancreas compared to healthy persons (respectively: 307.3 ± 128.2 A.U (arbitrary units – units of optical density); 188.2 ± 75.7 A.U., p < 0.01). The change of distribution of cells producing PP in tissue in smoking CP patients was observed. These cells was noticed in area of whole island and in among acinar cells.

Conclusion: Although the study results are presently inconclusive and potentially contradictory, the findings are nevertheless encouraging, and indicate that PP might have a role in diagnosis of pancreatitis. Further data and prospective controlled studies are needed to judge whether PP is of clinical value for diagnosing, staging and predicting long-term outcome in chronic pancreatitis or/and diabetes.
Development of protective chimeric antibody against tick-borne encephalitis virus

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Tick-borne encephalitis virus (TBEV) is a pending problem in European countries as well as Asian part of Russia and Kazakhstan. The most effective ways to treat this disease are vaccination and etiotropic therapy by serum immunoglobulin, obtained from donors living at the natural area of TBEV. However, serum immunoglobulin is unsafe and difficult to obtain. That's why researchers and pharmaceutical factories concentrated their efforts on design of recombinant antibodies against TBEV.

Chimeric antibody was constructed on the basis of mouse monoclonal antibody capable to neutralize TBEV. Two plasmid DNAs encoding light and heavy chains of recombinant antibody were constructed and used to co-transfect eukaryotic cells CHO-K1. Chimeric antibody purified by affinity chromatography was proven to bind recombinant analogue of TBEV glycoprotein E with the affinity about 0.06 nM, as was shown by surface plasmon resonance-based system. Mass-spectrometry analysis showed that recombinant antibody is glycosylated, has correct size, and its sequence corresponds to initial genetic constructions. Experiments carried out on mice provided that the antibody is capable to protect mice against TBEV with high efficiency.

Active caspase-3 is stored within secretory compartments of mast cells

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Caspase-3 is a main executioner of apoptotic cell death. The general notion is that, in viable cells, caspase-3 is mainly found as an inactive pro-enzyme present in the cytosol and that caspase-3 activation is largely confined to processes leading to cell death. Here we challenge this notion by showing that active caspase-3 is stored in viable mast cells and we also show that activation of mast cells, with preserved cellular viability, causes extracellular release of caspase-3 accompanied by a depletion of cell-associated caspase-3. During the course of mast cell differentiation from bone marrow cells, pro-caspase-3 was present in cells of all stages of maturation. In contrast, active caspase-3 was undetectable in bone marrow cells but increased progressively during the process of mast cell maturation, its accumulation coinciding with that of a mast cell-specific secretory granule marker- mouse mast cell protease 6 (mMCP-6). Whereas detergent concentrations sufficient for solubilization of cytosolic protein failed to solubilize active caspase-3, caspase-3 was solubilized after increasing the detergent concentration to levels sufficient for solubilization of secretory granule-specific proteases. Moreover, active caspase-3 was rapidly released into the cytosolic compartment by secretory granule-permeabilizing agents. Using a cell-permeable probe for caspase-3, the presence of active caspase-3-like activity in granule-like compartments close to the plasma membrane was demonstrated, and it was shown that mast cell activation caused release of the caspase-3-containing granule-like structures to the cell exterior. Together, the present study suggests that active caspase-3 can be stored within secretory compartments of mast cells.
SW05 Biochemical Mechanisms of Immune Defense

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in vivo fractions of macrophages showed that Lf binds to the IL-6 pro-

chromatin immunoprecipitation assay. Our results on nuclear

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fellowships awarded to Paula Florian.

This work was supported by CNRS-USTL/Institute of Biochemistry, and FEBS and EFIS-IL internalisation de la recherche standing of the Lf immunomodulatory mechanism.

more, we have investigated in situ binding of Lf on LfRE using a chromatin immunoprecipitation assay. Our results on nuclear fractions of macrophages showed that Lf binds to the IL-6 promoter in vivo.

Our results might bring more insights into the current understand-

of the Lf immunomodulatory mechanism.

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Reference


SW05.S23–8

Antibodies against ectromelia virus capable of neutralizing variola virus: generation and application for epitope mapping

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Virus-neutralizing antibodies are very important in host defense against viral infection. One possible approach is the selection of therapeutic antibodies against orthopoxviruses, naturally occurring in immunized donors using phage display methods. Development of antibody against variola virus and monkeypox virus is accompanied by necessity work in BSL 3-4 biosafety level, which leaves many limitations and is resource-intensive. Using of non-pathogenic virus in the first stages of recombinant antibodies selection would significantly simplify, reduce the cost, and, possibly, speed up the creation of therapeutic antibodies. It is known that the surface proteins of orthopoxviruses is highly homologous and in response to infection various orthopoxviruses each animal models produce enough similar pattern of specific antibodies. Given this, we used non-pathogenic to humans ectromelia virus (ECTV) for selection phage antibodies (pdAbs) against the variola virus (VARV).

Five unique pdAbs against ECTV was isolated from a combinatorial phage display library of human scFv antibodies constructed from the Vh and Vl genes cloned from the peripheral blood lymphocytes of vaccine-immune donors. The specificity of selected pdAbs was confirmed by ELISA, AFM and Western blot analysis. Plaque-reduction neutralization tests showed that variola virus was neutralized by selected pdAbs. One pdAb displayed a dose-dependent neutralization for different orthopoxviruses.

Target protein for neutralizing pdAbs was shown to bind a protein with a molecular mass of approximately 35 kDa, corresponding to the immunodominant protein in the development of orthopoxvirus infections in humans. This protein is encoded by open reading frame H3L (vaccinia virus, strain Copenhagen). To determine an epitope, responsible for binding with neutralizing antibodies, panel of p35-truncated proteins was constructed based on a putative 3D structure, antigenic and hydrophobicity index of p35 protein. The ability of the selected anti-ECTV pdAbs recognized truncated p35 protein was investigated by Western blot analysis. The antibody was shown to recognize only two truncated p35 proteins.

Thus, in this report the possibility of selection anti-VARV antibodies using non-pathogenic ECTV as an antigen for biopanning and screening was revealed. Target protein for neutralizing pdAbs was defined and epitope was defined.

SW05.S23–9

Dendritic cells as pre-exposure and post-exposure antitumor vaccines

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Anti-tumor vaccines on the base of dendritic cells (DCs) are perspective tools for tumor prophylaxis and therapy due to their specificity towards tumor cells, their ability to prevent systemic metastasis and to provide long-lasting anti-tumor effect. Moreover, this set of properties of DC-based vaccines provides for efficient prevention of tumor recurrence. The most common way of anti-tumor immunotherapy is the use of DC as a post-exposure vaccine in the case of already established tumors whereas the use of DCs as a pre-exposure antitumor vaccine is more problematic and depends on the specialties of patients. Such patients may include people with predispositions to cancer, for instance women carried mutations in BRCA1 and BRCA2 genes and men with elevated levels of prostate-specific antigen in the blood have significant risks of breast and prostate cancer development, respectively.

We compared two treatment schemes with DCs that display the models of pre-exposure and post-exposure vaccination. Three different experimental murine tumor models were used: adenocarcinoma Krebs-2 (primary tumor node), melanoma B16 (metastatic tumor model without primary node) and Lewis lung carcinoma (LLC) (metastatic tumor model with primary node). Dendritic cells generated from bone marrow-derived DC precursors were loaded with total RNA isolated from respective tumor cells and were used for vaccination. Post-exposure vaccination with DCs was the most efficient for Krebs-2 and melanoma B-16. We observed 1.8-2-fold retardation of tumor growth and 3-4-fold suppression of metastases number. Pre-exposure vaccination with DCs gave positive outcome in LLC model, where 4-fold suppression of metastasis was observed.

The success in application of DCs as pre-exposure vaccines allows suggesting them as possible cure for patients with genetic predisposition to cancer.

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Currenty, there are more studies of celiac disease, which is immune-mediated intolerance to alpha-gliadin – the main protein of gluten grains. It is known that immunotoxicity response intestinal mucosa of celiac disease is caused by a modification of gliadin, forced on it by tissue transglutaminase (TG).

Transglutaminase (TG, protein-glutamine \(\gamma\)-glutamyl transferase, EC 2.3.2.13) catalyzes the transamination reactions that are of greatest interest to the processing of food materials. Transamination – is acyl transfer between the \(\gamma\)-carboxyamide group of peptide-bound glutamine residues (acyl donors) and various primary amines (acyl acceptors), including the e-amino groups of lysine residues. The reaction can be both intra-and intermolecular, in the latter case, it leads to an increase in the molecular weight of the protein molecules according to the scheme:

\[
R_1\text{-Glu-CO-NH}_2 + H_2N-R_2 \rightarrow R_1\text{-Glu-CO-NH-R}_2 + NH_3
\]

In addition to the reactions of transamination TG catalyzes deamination of glutamine residues with the formation of glutamic acid. In some cases, water serves as the acyl acceptor. It can be assumed that such reactions occur mainly in the absence of a suitable substrate:

\[
R_1\text{-Glu-CO-NH}_2 + H_2O \rightarrow R_1\text{-Glu-COOH} + NH_3
\]

Deamination of glutamine residues results in a product that has an affinity to HLA DQ2 or HLA DQ8 in B-lymphocytes, and is able to activate the proliferation and differentiation of plasma cells. Consequently synthesized specific antibodies (anti-gliadin antibodies (AGA), anti-TSH (ATTG) and endomysial (AEMA)), which leads to the manifestation of celiac disease and atrophy of the small intestine wall.

A gluten-free diet is still considered to be the best outcome for patients with this disease. However, recent research in the field of technology products of special meals are focused on finding new ways to solve this problem by modifying the gluten in different ways. In this regard, MTG industrial application has been developed [1].

Along with its technological influence MTG alters the functional properties of proteins, it was found that the enzymatic modification of gluten decreases its binding to specific antibodies [6]. A number of studies found that the deamination of gluten increases its immunogenicity [2,4]. However transamination ability MTG was much higher than deamination [5]. Recent studies have confirmed a significant reduction in gluten immunotoxicity under the influence of the drug MTG [3]. Thus, our data and published ones showed mixed results, obtained by studying the effect of TG on different substrates and their combinations in different model conditions. Based on the foregoing, we conclude that the need for targeted, well-controlled studies of substrate specificity of TG and immune activity of the resulting products.

References

New approach for purification native NS1 protein of tick-borne encephalitis virus, promising for development new ELISA

Tick-Borne Encephalitis’s diagnostics system

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Tick-borne encephalitis (TBE) is a member of the Flaviviridae family of RNA viruses. TBEV NS1 protein is a glycoprotein secreted during virus replication. Serum of TBE patient is known to contain antibody against TBE virus NS1 protein. This protein being non-structural protein, serum of immunized patient isn’t contained immunoglobulin against this protein. It was reported that some patient was infected by TBE virus after vaccination. This case of TBE virus infection could be detected using ELISA test system to serum immunoglobulin against TBE virus NS1 protein.

To develop a panel of monoclonal antibodies against TBE virus NS1 protein, a new approach to purify NS1 protein was designed. NS1 protein was purified from inactivated virus-containing cultural medium using affinity chromatography. In electrophoresis analysis, it was shown that purified NS1 protein has three forms: monomer (~40 kDA), dimer (~80 kDA), and trimer (~120 kDA). Specific human and mice anti-TBE virus serum was shown to recognize all forms of the TBEV NS1 protein in ELISA and Western blot analysis. Other TBEV proteins have not indicated. It was suggested that purified NS1 protein could be used to immunize mice to produce high-affinity monoclonal antibodies against the TBEV NS1 protein and to develop ELISA test system. After purification TBE virus NS1 protein panel of monoclonal mouse antibody was designed. Selected monoclonal antibodies were nanomolar affinity.

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activity. Furthermore, mast cells contain proteases to facilitate the inflammatory responses in hypersensitivity and allergic reactions. A serine protease inhibitor, Kazal-type 5 (SPINK5) gene encoding LEKTI has been reported to play a suppressive role in atopic skin diseases. Interestingly, differentiating human T helper 2 (Th2) cells have also shown elevated expression levels of a serine protease inhibitor kunitz-type 2 (SPINT2). However, an in depth understanding how protease inhibitors potentially modulate allergic reactions in atopic diseases has remained largely unknown.

This study aims at characterizing the expression and secretion of SPINT2 from differentiating and fully polarized human effector Th2 cells as well as identifying the specific gene expression patterns of SPINT2 displaying Th2 cells.

We used naïve human CD4+ T cells isolated from umbilical cord blood and activated them without (Th0) and with cytokines IL-4 or IL-12 to induce differentiation of Th2 and Th1 effector cells, respectively. The kinetics of SPINT2 expression was detected both at RNA and protein level along the differentiation process by qRT-PCR and immuno-labeling and flow cytometry. To examine the cell subset specific gene expression profiles the differentiating Th2 cells were sorted at 24 h time point post activation based on their SPINT2 surface expression and the RNA was extracted for Affymetrix arrays.

Th2 cells expressed significantly more SPINT2 both at RNA and protein levels throughout the differentiation process compared to other Th subsets. SPINT2 was also secreted to the culture medium from Th2 cells in increasing amounts towards fully polarized Th2 cells. With the genome wide gene expression analysis we found 97 uniquely expressed genes in SPINT2 positive cells polarized Th2 cells. Several of these genes are linked to natural killer cell associated cytotoxic pathways but include also chemokine genes related to asthma and migration of Th2 cells, e.g. CCL5 and CCL22. The results are expected to gain new important information on serine protease inhibitor exploitation of Th2 cells. The interleukin-2 receptor alpha (IL-2Ralpha) in association with IL-2Rbeta and IL-2Rgamma forms a high affinity IL-2R that is transcription level by antigen and cytokine. In primary human T cells the role of STAT5 phosphorylation, cell surface expression of CD25 and lymphopenia is mediated by extracellular signals.

Constitutive STAT5 phosphorylation is critical for interleukin-2 receptor alpha expression in human blood T lymphocytes

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The interleukin-2 receptor alpha (IL-2Ralpha) in association with IL-2Rbeta and IL-2Rgamma forms a high affinity IL-2R that is important for normal T cell function and proliferation. Expression of the IL-2Ralpha gene by T cells is regulated mainly at the transcription level by antigen and cytokine. In primary human blood T cells we used pharmacological drugs to separate distinct signal pathways which are involved in IL-2Ralpha expression (assayed by flow cytometric analysis of CD25+ cells) and showed that the Src-dependent signalling (via TCR) preceded the Jak-dependent signalling (via IL-2R) during cell surface expression of CD25. Since recombined IL-2 was revealed to induce CD25 expression in competent (not in quiescent) T cells, the role of STAT5 signal delivered downstream of IL-2R was elucidated. Western blot analysis showed that STAT5 protein was unphosphorylated in quiescent lymphocytes. The tyrosine phosphorylation of STAT5 appeared only after 2 h of phytohemagglutinin (PHA) stimulation and high phosphorylation of STAT5 is observed during the next 48 h. In quiescent blood lymphocytes IL-2 induced short-term increase in STAT5 phosphorylation, whereas in the competent cells STAT5 phosphorylation was induced by IL-2 at 30 min and was kept at a high level during the next 48 h. WHI-P131, an inhibitor of Jak3 kinase, prevents STAT5 activation, cell surface expression of CD25 and lymphocyte proliferation. A correlation between alterations in tyrosine phosphorylation level of STAT5 and the expression of CD25 was established in T lymphocytes stimulated by PHA or IL-2. It is concluded that Jak3/STAT5 signalling via IL-2 receptor is nesse-
ELISA for detection of 19-nortestosterone in dietary supplements

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Competitive enzyme-linked immunosorbent assays (ELISA) were developed for detection of 17β-19-nortestosterone (19-nortestosterone, NT) and its esters in dietary supplements. 19-nortestosterone and its esters are frequently missed anabolic steroids in the food industry (e.g. cattle) as well as in the doping in sports and fitness. Conjugates of 19-nortestosterone (3-O-carboxymethyl)oxime (NT-3-CMO) and 19-nortestosterone 17-O-hemisuccinate (NT-17-HS) with bovine serum albumin were used as immunogens. The assays are based on the competition between immobilized conjugates of NT-3-CMO or NT-17-HS with ovalbumin and the respective polyclonal rabbit antiserum. The degree of competition was measured by the use of the secondary antibody labeled with peroxidase followed by a color reaction with tetramethylbenzidine. The detection limits and the fifty percent intercept (I50) for NT-3-CMO ELISA were 0.01 ng/ml and I50 = 6.4 ng/ml; and for NT-17-HS ELISA were 0.01 ng/ml and I50 = 1.4 ng/ml. The NT-3-CMO antiserum had a few cross reactions, with main cross-reactants being testosterone and dehydroepiandrosterone. The NT-17-HS antiserum had significantly better cross-reactivity, mainly with the esters of 19-nortestosterone.

An immunochromatographic strip test for a rapid detection of anabolic steroid 19-nortestosterone

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19-Nortestosterone (17β-Hydroxy-19-norandrost-4-en-3-one), also named nandrolone, is an anabolic steroid which occurs naturally in the human body, but only in tiny quantities. It is very similar in structure to the male hormone testosterone. Ergogenic use for this steroid in sports, racing, and bodybuilding is controversial because of its adverse effects and the potential to gain an advantage. Its use is referred as doping and banned by all major sporting organizations.

Compared with traditional instrumental methods usually used for analysis of anabolic steroids, immunoassays are portable and cost-effective, with adequate sensitivity, high selectivity, and a simple sample extraction process. Therefore, immunochemical techniques have become popular and are increasingly considered as alternative/complementary methods for residue analysis.

This study successfully demonstrated the potential of using the rapid immunoassays for sensitive detection of 19-nortestosterone in nutritional supplements. Highly specific rabbit polyclonal antibody and the nortestosterone-3-OVA conjugate were used for construction of a gold particle-based immunochromatographic strip test. Under optimized experimental conditions the visual LOD was 1 ng/ml. The developed test is sensitive enough and it will be useful for incorporation into monitoring programs for the control of food supplements contamination.

Ultrastructural studies on CD14 distribution in LPS-stimulated macrophages

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Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria which strongly induces pro-inflammatory responses of macrophages. Initially, LPS is bound to CD14 protein which transfers LPS to a signaling complex of toll-like receptor 4 (TLR4). Activated TLR4 triggers two pro-inflammatory signaling pathways which engage MyD88 or TRIF adaptor proteins. Taking into account a crucial role of CD14 in LPS-induced responses, we undertook confocal and electron microscopy studies on CD14 distribution in macrophages.

Confocal microscopy analysis revealed that CD14 occupied the plasma membrane surface but was present also in cytoplasmic vesicles in J774 cells. A part of these vesicles colocalized with PI (4)P which suggested their Golgi origin. To get insight into CD14 distribution in the plane of the plasma membrane, large sheets of the membrane were obtained by mechanical cleavage of cells and subjected for immunogold labeling and ultrastructural analysis. We found that stimulation of cells with 100 ng/ml LPS induced transient clustering of CD14 in the plasma membrane reflected by a shift of CD14-attributed gold labels from singlets to aggregates of more than 10 particles. In addition, numerous vesicles rich in CD14 fused with the plasma membrane during LPS action. In these vesicles, caveolae but not clathrin, was also found. Biochemical analysis indicated that CD14 was enriched in raft fractions of the plasma membrane at the onset of cell stimulation with LPS. The CD14-rich vesicles were devoid of MyD88, which associated with the plasma membrane in LPS-stimulated cells and colocalized with CD14 distributed outside of the vesicles. We assume that the vesicles bring new pools of CD14 to the plasma membrane in LPS-stimulated cells while MyD88-containing signaling complexes of TLR4 are formed outside of these structures. LPS-induced incorporation of CD14 to the plasma membrane is likely to be local and does not affect substantially the amounts of CD14 on the surface level, as indicated by flow and laser scanning cytometry analysis.

The development of a sensitive immunoassay for the detection of new synthetic drugs

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New synthetic psychoactive drugs are the most destructive and available compounds today. Older drugs as heroin or cocaine destroy person’s life but new drugs are more dangerous because these compounds not only cause overdose, a psychotic episode of hallucinations, aggression, impulses and homicidal tendencies. Using of these drugs is widening amongst young people. Consequently, there is growing public interest in development of versatile detection methods enabling to effectively intervene in this field.
Traditional methods of analysis of narcotics are gas and liquid chromatography – GC/MS, LC/MS. Their advantages are high sensitivity and reliability. On the contrary, instrumental methods are less rapid and they involve multiple steps in sample preparation and analysis, require expensive equipment and skilled analysts, and thereby they are unsuitable for routine analysis of a large number of samples or on-site determinations. An alternative approach is represented by immunoassays which could be portable and cost-effective, with adequate sensitivity, high selectivity and a simple sample processing.

In this work, enzyme-linked immunosorbent assays have been developed for the detection of selected synthetic cathinones. Carbamoylated derivatives of parent drugs were prepared as hapten for synthesis of immunogens and subsequently a series of polyclonal rabbit antibodies were prepared. The antibodies and haptenes were used as immunoreagents in competitive Enzyme-linked immunosorbent assay. Under optimal experimental conditions, the most sensitive assay achieved IC50 and limit of detection values of 2 ng/ml and 0.09 ng/ml, respectively.

We conclude that after the validation for individual matrices, these methods enable effective fast detection of synthetic cathinones in operational conditions.

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**SW05.S23–19**

**Immunodetection of human CA XII as a new potential biomarker of tumor cells**

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Proposed objective: Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc enzymes that catalyse the reversible hydration of carbon dioxide to bicarbonate. CAs have important physiological and pathological functions in human organism. Some human CAs, such as CA IX are well-recognized tumor markers. Recently, the association of CA XII with human cancers has been demonstrated. The CA XII is a transmembrane protein with an extracellular catalytic domain. It is involved in tumor progression by acidification of the extracellular milieu and regulation of intracellular pH. Recent studies indicate that CA XII is aberrantly overexpressed in breast, cervix, brain cancers, renal carcinomas. Therefore, CA XII might be considered as a useful biomarker of tumor cells and a promising target for specific therapies. The aim of the current study was to develop new monoclonal antibodies (MAbs) against human recombinant CA XII and evaluate their diagnostic potential.

Materials and Methods: The extracellular domain of human CA XII was expressed in *E. coli* and used as an immunogen. The MAbs were generated by hybridoma technology. MAb specificity was analysed by ELISA, IHC and flow cytometry.

Results: Seven stable hybridoma cell lines producing IgG antibodies against human CA XII were generated. The MAbs were highly specific to CA XII and did not cross-react with human recombinant CA I, II, III, VII, and XIII. In order to demonstrate the diagnostic value of the MAbs, they were employed for the IHC staining of CA XII in human tumor tissue specimens. The MAbs demonstrated a strong and specific reaction with colon and renal carcinoma specimens and did not show any unspecific background staining of the respective normal tissues. Flow cytometry analysis revealed a specific immunostaining of CA XII in human tumor cell lines.

Conclusion: The newly developed MAbs represent a promising diagnostic tool for the immunodetection of CA XII-expressing tumor cells.

**SW05.S23–20**

**Effect of enzymatic cross-linking on allergenic properties of bovine beta-lactoglobulin**

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β-Lactoglobulin (BLG) is a whey protein implicated in cow’s milk allergy (CMA). Currently, enzymatic cross-linking of proteins, including allergens, is exploited in food processing. The number and nature of protein cross-links during and after food processing offers a means by which the food industry can manipulate the functional properties of food, often without damaging the nutritional quality. The aim of the present study was to compare native and cross-linked BLG in trans-epithelial transport and allergenic potential.

Cross-linking of BLG resulted in formation of highly polymerized, fiber-like adducts of molecular mass above several MDa. When comparing epithelial transport in a Caco-2 cell-line monolayer, cross-linked BLG was transported up to 100 times slower than native BLG. *In vivo* transport studies in mice revealed that cross-linked BLG had longer half-life in the gut and was found both in villi and Peyer’s Patches in contrast to native BLG which was detected in villi only. Interestingly, considerable differences between native and cross-linked BLG were observed in the induction of food allergy in a mouse model. Feeding cross-linked BLG resulted in higher IgG1 and IgE antibody levels, although the levels of mast cell degranulation after allergen challenge did not increase.

In *ex vivo* T cell restimulation, levels of INF-γ did not differ among the groups, but significant IL-13 production was observed only in spleen cultures from the cross-linked BLG sensitized mice.

From these studies it can be concluded that enzymatic cross-linking has increased the immunogenic potential of BLG, probably due to its particulate size, repetition of protein epitopes, prolonged half-life and uptake through the Peyer’s Patches in the intestine. This may have consequences for the use of these proteins in food processing.

**SW05.S23–21**

**Impact of caffeine in the internalization of Candida albicans by human keratinocytes**

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*Candida albicans*, a member of the normal human microbiota, is an agent of systemic disease with high morbidity and mortality. The skin infection, although not life threatening, is considered a risk factor for development of severe infections, especially in immunodepressed. Keratinocytes are skin cells, not professional phagocytic cells but that can endocytose microbes, which subsequently cross to subcutaneous layers. Furthermore, keratinocytes secrete factors, which recruit cells of the innate immune system thereby contributing to the elimination of emerging infections. Among these factors are ATP and adenosine, which play an important role in immune homeostasis, mediated by specific receptors. ATP acts as a danger signal, while adenosine and its receptors, particularly adenosine A2AR, play a protective role in the host cell damage due to immune-inflammatory response. Caffeine, an adenosine receptor antagonist, is used in cosmetic prep-
The results showed that caffeine reduces the internalization of C. albicans by keratinocytes and changes the keratinocyte morphology. It also had an impact on the extracellular concentration of ATP, decreasing this factor, known to be a flag of hazard. During C. albicans infection the extracellular concentration of ATP was lower than that observed when keratinocytes were stimulated with LPS, used as a control.

The expression of A2AR gene after infection with C. albicans was lower than that recorded after the stimulation with LPS. The expression of the genes of proinflammatory cytokines, IL-1β and TNF-alpha, showed that the keratinocytes had a delayed response to LPS and to C. albicans infection, in what regards the expression of these genes. The introduction of caffeine during stimulation with LPS or during infection by C. albicans did not result in statistically significant differences.

In conclusion, this study demonstrated that the presence of caffeine could alleviate or prevent the keratinocyte infection by C. albicans. Further studies will unveil the exact molecular mechanisms by which this drug changes the ability of these cells to internalize C. albicans.

**SW05.S23–22**

**Peptide selection for binding to MHC class I: insights from structure and molecular dynamics**

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Major histocompatibility complex (MHC) class I molecules present antigenic ligands derived from endogenous proteins for T cell surveillance. Different MHC class I alleles bind different repertoire of peptides with anchor residues that fit into the narrow pockets of the MHC peptide-binding groove. Polymorphism of MHC class I pockets determines which peptides can bind.

This concept has been unchallenged for over two decades. We found that peptides with side chains expanding beyond the size of anchor residues could still fit in the MHC class I peptide-binding groove. We crystallized such complexes and found that these peptides can be accommodated in MHC class I (H-2Kb) by generating particular alterations in the size of the pocket. This affects thermal stability of the peptide-MHC class I complexes. We also resolved dynamics of MHC class I pockets during peptide binding using fluorescence polarization-based assay for peptide on- and off-rate measurements and molecular dynamic simulations.

Low temperature can stabilize binding of low affinity peptides with altered anchor residues to MHC class I molecules. We are currently investigating presence and abundance of such non-optimal peptides in MHC class I peptideosome using large-scale proteomic approach on cells cultured at low temperatures as compared to physiological temperature.

Our work suggests that MHC class I molecules are less discriminative in binding different peptides as generally accepted. Peptide binding to MHC class I is followed by an induced fit for the pockets accommodating the anchor residues. After a stability check at 37°C, peptides with expanded anchor residues are released while peptides with canonical anchor residues remain in the peptide-binding groove inducing conformational change. Such MHC class I-peptide complexes are presented at the plasma membrane. Knowledge on MHC class I peptide binding will have further application in the ensuing response and effective immunotherapy.

**SW05.S23–23**

**Quantitative study of mono-and multivalent interactions between viral antigens and antibodies or their derivatives of different compositions**

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Due to bivalence of IgG antibodies their interaction with multivalent antigens leads to the formation of a number of complexes having various affinity and stoichiometry. This variability affects the regulation of the processes initiated by the immune recognition, as well as the efficiency of immunoassays and other biotechnological systems. However, the dependence of these effects on the size and valence of antigen and antibody preparations was described only empirically. The aim of this study was to quantify the relative characteristics of the interactions between viral antigens and antibodies or their derivatives having different structures and valences. A series of preparations were obtained, including monoclonal antibodies to potato virus X and plum pox virus, their Fab fragments and antibody conjugates with colloidal gold nanoparticles with diameters from 6 to 50 nm. The formation of immune complexes with the obtained preparations was studied by flow cytometry, transmission electron and atomic force microscopy. The Biacore biosensor system was used to measure the equilibrium and kinetic constants of the immune interactions. A 50–100-fold increase in the affinity was shown for the transition from the Fab fragments to bivalent IgG antibodies. A monotonic increase in both the equilibrium and kinetic constants was found for a series of antibody conjugates with nanodispersed carriers of varying diameter. In the size range under study, these values differ by two orders of magnitude, and the dependence on the particle diameter is stronger than the dependence on the surface density of the immobilized antibodies. To quantify individual molecular interactions, single-molecule force spectroscopy was implemented. The analysis was carried out using the adsorption of the antigen on the substrate and by recording the rupture force for the cantilever with the immobilized antibody. It is shown that the force distribution has statistically reliable peaks of nonspecific adsorption (average rupture force is 110 ± 30 pN), monovalent interactions (average rupture force for antibodies to plum pox virus is 230 ± 90 pN) and bivalent interactions (390 ± 70 pN). The factors affecting the variability of rupture forces for the complexes of the same composition were analyzed.

**SW05.S23–24**

**Probing the mechanism of action of the Pro-rich antimicrobial peptide Bac7 – an anti-infective and bacterial cell penetrating agent**

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Bac7, a bovine cathelicidin, is an antimicrobial peptide (AMP) characterized by a high content of Pro residues, and is selectively active against Gram-negative species, particularly Enterobacteriaceae. Unlike peptides from other AMP classes that have a principally membranolytic activity, Pro-rich AMPs (PR-AMPs), which are found in both insects and mammals, act
without an initial damage to bacterial membranes. Their mode-of-action rather requires penetration of the outer membrane, transport through the cytoplasmic membrane by a specific transport system (the inner membrane protein SbmA in *E. coli*) and interaction with internal targets leading to bacterial inactivation. One or more of these steps involves stereoselective interactions, as all-D Bac7 is much less active than the all-L natural form. Bac7 and other PR-AMPs are thus examples of cell penetrating peptides (CPP) for susceptible Gram-negative bacteria, with both intrinsic antibacterial activity and potentially the capacity to internalize otherwise impermeant cargo molecules into these bacterial cells.

The mechanism of action of Bac7 was studied using genetic and biochemical approaches, as well as specific bacterial deletion mutants, to characterize the transport machinery, intracellular targets, and to assess the role of the outer membrane barrier. Furthermore, structure/activity relationship studies using fluorescently labelled Bac7 fragments of different lengths have allowed identifying the minimum sequence requirements for efficient internalization into bacteria and to correlate this with the minimum antimicrobial requirements. These studies have indicated a particularly important role for N-terminus of Bac7, which is rich in arginine residues. By substituting key Arg residues with natural (Lys) or non-proteinogenic (D-Arg, citrulline, methylated or nitro-Arg) or peptoid (N-Arg) analogues, we have been able to probe the role of structural and physico-chemical features in mediating transport and/or cidal activity. Results indicate that Bac7 may be internalized using an integrated transport system involving inner and outer membrane components. Finally, a Bac7 fragment was capable of protecting animals against bacterial infections with remarkably low toxicity, although its clearance was rapid.

**SW05.S23-26**

**Study of structures formed by FtsZ in *Escherichia coli* and Mycoplasma hominis cells**

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FtsZ, a prokaryotic homolog of tubulin, was found in almost all prokaryotes including Mollicutes (mycoplasmas), the simplest self-replicating organisms. The main function of FtsZ is participation in cytokinesis. FtsZ is considered to be a major component of Z-ring, which is formed at the septum between two dividing cells. Study of division in mycoplasma will allow us to gain knowledge of cytokinesis basic principles. Immune-electron microscopy of human pathogen mycoplasma, *M. hominis*, showed that FtsZ is mainly localized in septa between dividing cells and in tethers between divided but not yet completely separated cells. Some evidence of spiral structures (Z-spirals) has also been found. Unfortunately, limitations of the method didn’t allow us to draw definitive conclusions about mycoplasma Z-structures. Therefore we decided to use super resolution localization microscopy.

We have constructed and tested an optical set-up to perform astigmatism-based 3D localization microscopy. One of the main issues of localization microscopy is sample drift. To prevent it we created an active feedback system that measures the position of polystyrene bead attached to the coverglass using infra red laser beam and a quadrant photo detector and compensates the drift by piezo stage movement. This system stabilizes sample position in 3D with precision of about 10 nm. Introduction of cylindrical lens into the optical path of the microscope allows to measure z-positions of single molecules with the precision of 50 nm. Genetic engineering of *M. hominis* is a challenging task so immunocytochemistry was chosen to visualize FtsZ in these cells. To achieve temporal separation of fluorescence we tested a number of dyes: Rhodamine-6Gg, ATTO-532 and Abberior CAGE 552. The latter was found to work best in our hands, being the most robust and flexible. Preliminary experiments proved our ability to visualize FtsZ structures in both *E. coli* and *M. hominis* cells. Further experiment should allow us to determine the role of FtsZ in *M. hominis* cells and validate the results obtained in *E. coli* with the use of FtsZ-mEos2 fusion protein.
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SW05.S23–27
Methods of molecular biology and biochemistry for learning about microorganism: comparison of C. sakazakii and C. malonaticus
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Cronobacter spp. are Gram negative opportunistic food-borne pathogens causing serious neonatal infections. This bacterial genus belongs to the family Enterobacteriaceae and currently comprises of seven species: C. sakazakii, C. malonaticus, C. universalis, C. turicensis, C. m Yuktensis, C. dublinensis, and C. condimenti.
In this study, strains of C. sakazakii and C. malonaticus were characterized using different methods. Firstly the panel of biochemical properties was tested for each strain. Further the restriction fragment length polymorphism (RFLP) patterns of rpoB gene and O-antigen gene cluster were used to follow differences within tested strains. The results were completed by species specific PCR. Obtained data were evaluated with respect to the strain’s epidemiologic background (if known).
Mostly, the obtained results corresponded with current knowledge. Biochemical testing paralleled with species specific PCR in majority of strains and the rpoB clustering did not shown significant dissimilarities within both C. sakazakii, or C. malonaticus strains. During this study, three previously described serotypes (O1, O2, and O6) were successfully identified in Cronobacter sakazakii and Cronobacter malonaticus. However in 3 strains atypical combination of biochemical properties together with rph profile were observed. It was found, these strains were isolated from the same type (milk powder) of sample (in different countries – USA, Russia and the Netherlands). Further, the relevance of obtained information will be studied with respect to possible virulence of targeted strains (sequence type, protein profile).
This work was supported by the Czech Grant Agency (project No. P503/12/P704 and project No. 13-23509S).

SW05.S23–28
Toll-like receptor 2 participates in the internalization of Staphylococcus aureus stimulated by prolactin in bovine mammary epithelial cells
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Bovine mammary epithelium (bME) is involved in mastitis, which is involved in the innate immune modulator in mammary gland, which leads to a better defense against bacterial infection.

Vitamin D has immunomodulatory functions regulating the expression of host defense genes. The aim of this study was to determine the effect of cholecalciferol (vitamin D3) on S. aureus internalization into bovine mammary epithelial cells (bMEC) and antimicrobial peptide (AP) mRNA expression. Cholecalciferol (1–200 nM) did not affect S. aureus growth and bMEC viability; but it reduced bacterial internalization into bMEC (15–74%). Also, bMEC showed a basal expression of all AP genes evaluated, which were induced by S. aureus. Cholecalciferol alone or together with bacteria diminished tracheal antimicrobial peptide (TAP) and bovine neutrophil beta-defensin (BNBD) 5 mRNA expression; while alone induced the expression of lingual antimicrobial peptide (LAP), bovine beta-defensin 1 (DEFB1) and bovine psoriasin (S100A7), which was inhibited in the presence of S. aureus. Fifty nanomolar of cholecalciferol increased BNBD10 mRNA expression coinciding with the greatest reduction in S. aureus internalization. Genes of vitamin D pathway (25-hydroxylase and 1α-hydroxylase) show basal expression, which was induced by cholecalciferol or bacteria. S. aureus induced vitamin D receptor (VDR) mRNA expression, but not in the presence of cholecalciferol. In conclusion, cholecalciferol can reduce S. aureus internalization and differentially regulates AP expression in bMEC. Thus, vitamin D could be an effective innate immunity modulator in mammary gland, which leads to a better defense against bacterial infection.
**SW05.S23–30**

*In vitro* selection and evolution of DARPin displays using SNAP display

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Display systems have proved to be indispensable tools for isolating and evolving protein binders. Entirely *in vitro* systems for the selection of protein binders have been developed to avoid the shortcomings of cell-based systems such as phage, bacterial and yeast display. The relatively unstable nature of their genotype-phenotype linkage is however limiting the applicability of the two most widely used methods, namely ribosome and mRNA display. Recently, SNAP-display was developed to provide a more stable, covalent link between protein and coding DNA in water-in-oil emulsions. SNAP display has been shown to provide ~100 fold enrichment in model experiments [1].

The aim of my work is to use SNAP display to select and evolve protein binders from a library of designed ankyrin repeat proteins (DARPin) against the human epidermal growth factor receptor (Her2), a therapeutic target implicated in breast cancer. The dynamic range of the display system was tested with a previously isolated DARPin binder with picomolar affinity for Her2 [2]. Stringency conditions were optimised to decrease non-specific binding and enhance enrichment efficiency. After three rounds of selection against Her2 in the presence of a large excess of a negative control, the picomolar DARPin was enriched 102-fold. To mimic affinity maturation, the picomolar binder was competed against a published Her2 nanomolar binder [3]. Successful selection of picomolar over nanomolar indicated that, besides allowing the recovery of established Her2 nanomolar binder [3]. Successful selection of high-affinity binders from naive libraries, the system is also suitable for affinity maturation. We will use SNAP display for affinity selections and optimisation of binders via directed evolution.

References


**SW05.S23–31**

Amino acid substitutions in plant-produced conservative influenza antigen significantly increase animal immune response and protective efficacy of candidate nanovaccines

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A new approach for super-expression of the influenza virus epitope M2e in plants has been developed on the basis of a recombinant tobacco mosaic virus (TMV-U1) genome designed for Agrobacterium-mediated delivery into the plant cell nucleus. The TMV coat protein (CP) served as a carrier and three versions of M2e sequence were inserted into the C-terminal surface loop.

The set of viral vectors TMV-M2e was constructed with Cys-Ser or Cys-Ala substitutions at the positions 17 and 19 of the human consensus sequence of M2e epitope. Agroinfiltration experiments showed that the chimeric viruses were capable of spreading via vascular system of systemically infected Nicotiana benthamiana plants producing as much as 4 g of TMV-M2e-ala coat protein per 1 kg of fresh leaves in 2 weeks. Genetic stability of all recombinant viruses was demonstrated. Following the purification, TMV-M2e-ala particles contained up to 90% of CP-M2e fusion proteins in preparation. Antisera to chimeric viros appear to contain far more antibodies specific to influenza virus than those specific to TMV particle itself (ratio differs from 2.7/1 to 5/1 depending on substitutions in the amino acid sequences of epitopes). Immunogold electron microscopy proved that the foreign antigens were uniformly distributed and tightly packed on the surface of the recombinant TMV virions. Apparently, the majority of TMV CP epitopes in chimeric TMV-M2e are hidden from the immune system by the M2e epitopes. The profile of IgG subclasses after immunization of mice with TMV-M2e nanoparticles demonstrated a significant difference in the levels of IgG1 and IgG2a (IgG1/IgG2a ratio differs from 0.7 to 3.2). Mice immunized with the chimeric viruses were resistant to 5 lethal doses (LD) of the homologous influenza virus strain, A/PR/8/34 (H1N1). Alanine substitutions led to partial protection (5LD, 70% of survival rate) against a heterologous strain (6 amino acid changes comparing with the used M2e consensus) of influenza A/California/04/2009 (H1N1). This is the first successful production of a new form of rod-shaped chimeric viros where the density and proximity of conservative influenza M2e epitopes on the surface of rigid helical nanoparticles should be higher than on the surface of spherical, icosahedral or helical filamentous particles.

**SW05.S24 B Cells in Inflammation and Disease (V-W24)**

**SW05.S24–1**

Receptor-mediated signal transduction pathways that regulate B lymphocyte fate

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The regulation of B cell differentiation, proliferation, and apoptosis involves the integration of intracellular signals provided by receptors of innate and adaptive immune systems. During ontogeny B cells transit the several decision check-points where they are integrating many inputs that influence cell fate. B-cell receptor (BCR) and receptors of TNFR family are the key regulators of B cell fate. Signal transduction pathways initiated via these receptors regulate the transcriptional programs that establish the distinct B cell subset phenotype. The outcome of BCR ligation depends on its cooperation with signals from different co-stimulatory receptors. CD40, BAFF receptor, TACI, and BCMA provide co-stimulatory signals for BCR signaling to regulate both survival and apoptosis during immune responses. They coordinate germinal centre formation and Ig class switch recombination. CD150/SLAMF1 is also a co-receptor molecule that is involved in immune regulation. CD150 mediates different signal transduction pathways depending on the availability of downstream signaling elements, especially, the adaptor protein SH2D1A/SAP. All these receptors are linked to P38K-Akt/PKB, ERK1/2, p38 MAPK, JNK1/2 and PKD2 signal transduction pathways and are involved in regulation of transcription factor network that control B cell proliferation, differentiation and apoptosis. In addition, CD150 serves as entry receptor for measles virus that mediates immunosuppressive effects due to CD150 signaling properties. Signaling via cell surface receptors, which coordinate normal B lymphocyte differentiation, is important for lymphoma pathogenesis. Hallmark genetic abnormalities have been described in B-cell lymphomas, some of which happen during physiologic processes involved in the generation of immunologic diversity. Revealing the cell surface receptors that are involved in regulatory molecular networks will help to explore the design of novel therapies for B-cell lymphoproliferative disorders. Coordinated targeting of these pathways might provide a useful approach for treatment of candidate B cell lymphomas.
Abstracts

**SW05.S24–2**

B-cell derived microvesicles are important component of extracellular communication in health and disease

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The membrane vesicles secreted by many cell types have been widely detected in various biological fluids including peripheral blood, urine and ascitic fluids. Microvesicles may induce epigenetic changes in target cells by transferring bioactive molecules such as proteins, RNAs and microRNAs. Microvesicles (exosomes) derived from B cells exhibited MHC-II, B-cell receptor, various tetraspanins, immunoglobulin, adhesion molecules. The common pool of plasma microvesicles include microvesicles, secreted by various cells of blood. We isolated microvesicles from the plasma of treated CLL patients (n = 14) as well as healthy human subjects (n = 10) by differential centrifugation. The fractions of microvesicles, isolated after two consecutive centrifugations at 13 000 g and 100 000 g, were characterized by Western blot analysis using anti-CD63, Calnexin, GSTp, CD20 and beta-actin specific antibodies. The size and shape of distribution of vesicle preparations was estimated by electron microscopy. By using CD20 and CD19 antibody we identified B cell-derived microvesicles and CD20 and CD19 positive lymphocytes in healthy human subjects and CLL patients. Flow cytometric analysis demonstrated that B cell-derived microvesicles isolated from plasma healthy human subjects represent <5%. Notably, levels of B cell-derived microvesicles were variable in plasma of CLL patients. However, we did not observe any correlation between CD20 and CD19 positive lymphocytes and the levels of plasma microvesicles. For example, one patient with high level of CD20-positive lymphocytes (95%) in blood had 20% of CD20-positive microvesicles, while another patient with low number of CD20-positive lymphocytes (5%) had 45% of CD20-positive microvesicles. This could be explained by that formation of B cell-derived microvesicles could be influenced by a number of different factors, such as, for example, stage of disease and undergone treatment, and this, in turn, makes them a useful tool and prognostic indicator in personalized medicine. This study provides a methodology for isolation of microvesicles, their identification and quantification that is essential for development of microvesicles as a prognostic indicator of disease stage and efficacy of treatment.

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**SW05.S24–4**

The influence of long-term diet supplementation with biological active substances on level of inflammation markers in rat colon

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Inflammation and colon cancer are very important problem in human public health. One of the most effective methods of colon diseases prevention is diet and regular physical exercises. Chemoprevention with low dose of acetylsalicylic acid can be used as an effective method of lowering the risk of a further consequence of inflammation and cancer. Aspirin is not free from adverse reactions and could induce gastric haemorrhages or gastric ulcers. The use of aspirin is also forbidden for pregnant women, patients with haemorrhagic diathesis and aspirin-induced asthma. These adverse reactions exclude many patients from chemoprevention. That is why there is a constant need to search for alternative protective measures, which would be safe for the whole population. Colon inflammation markers are antiinflammatory cytokine interleukin 10 (IL-10, also known as human cytokine synthesis inhibitor factor and interleukin 12 (IL-12), proinflammatory cytokine involved in the differentiation of naïve T cells.

The aim of the present study was to evaluate the influence of complex diet supplementation with polyphenolic compounds, β-carotene, probiotics and n-3 and n-6 polysaturated fatty acids on the concentration of interleukin-10 and interleukin-12 in rats colon tissue. The following sources of bioactive substances used were: puree from pumpkin (*Cucurbita maxima*) as a source of β-carotene and prebiotics fiber, hydrolysed water extract from linden (*Tilia cordata*) inflorescence as a source of flavonoids, rapeseeds oil, salmon fat as a source of DHA, EPA and other
p-3 and n-6 fatty acids and two strains of bacteria with proven probiotic properties: *Lactobacillus acidophilus* LA-5 and *Bifidobacterium* *Animals ssp. lactis*.

Eight-week old Sprague-Dawley rats were fed for 12 months with standard maintenance of semi-synthetic diet enriched with above mentioned sources of bioactive substances. After 3 months first group was killed and examined.

The enzyme-linked immunosorbent assay (ELISA) analyses revealed that the diet supplementation with investigated bioactive substances caused statistically significant decreased level of interleukin 12 (p < 0.05). IL 10 level did not change in examined tissues. In second group killed after 12 month analysis revealed that level of IL 10 significantly increased (p < 0.05) and level of IL 12 (p < 0.05) significantly decreased compared to the control group. These data indicate that the risk of inflammation is lower for rats receiving a supplemented diet.

**SW05.S24–5**

Cholinergic regulation of B lymphocyte activation and antibody immune response

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B lymphocytes are regulated by a variety of soluble factors including cytokines, hormones and neurotransmitters like acetylcholine, which is also produced by immune cells. Correspondingly, they possess receptors to acetylcholine of both muscarinic and nicotinic type. We found that mouse B lymphocytes express several nicotinic acetylcholine receptor subtypes (nAChRs); the level of expression is genetically determined and depends on the state of B lymphocyte activation. By means of flow cytometry and sandwich ELISA it was shown that alpha4beta2 nAChRs are coupled to BCR, while alpha7 nAChRs, which limit B lymphocyte activation in response to both endogenous and T lymphocyte-produced acetylcholine. The use of B lymphocytes from alpha7–/– (BCR) in normal and malignant cases.

**SW05.S24–6**

Measles virus hemagglutinin affects CD150-mediated signaling in B lymphocytes and dendritic cells

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Measles virus (MV) constitutes an important health problem as it causes a profound immune system suppression leading to high morbidity and mortality. The major cellular receptor for wild type MV is CD150, which binds directly to MV hemagglutinin (MV-H) and is highly expressed on the main MV cellular targets – activated T and B lymphocytes and mature dendritic cells. CD150 modulates intracellular signaling triggered by the antigen receptors in B and T cells, but its ligation itself may also influence on several signal transduction pathways. To address the particular role of CD150-MV-H interaction in measles immunopathogenesis in the absence of infectious context, we created an experimental model of cells expressing wild type MV-H on their surface – CHO-H cell line. We demonstrated that the interaction with MV-H inhibited CD150-induced JNK1/2 and p38MAPK phosphorylation (activation), but not ERK1/2 phosphorylation in human B lymphocytes and dendritic cells (DCs). Moreover, MV-H stimulated Akt phosphorylation on S473 in both, B cells and DCs. We also revealed the downregulation of surface expression of CD150, CD80, CD83, CD86 and HLA-DR molecules on DCs, inhibition of IL-12, but not IL-10, production, and suppression of DC-mediated T-cell alloproliferation. Engagement of CD150 by MV-H in mice transgenic for human CD150 decreased the inflammatory response, confirming the immunosuppressive effect of CD150-MV-H interaction in vivo.

Altogether, the obtained results reveal a novel mechanism of MV-induced immunosuppression and extend our knowledge about immunomodulatory role of CD150 receptor. Our studies represent an important issue for the understanding and preventing the disease complications after acute phase of measles infection.

**SW05.S24–7**

Protein kinase D2 as a marker of differentiation of normal and malignant human B lymphocytes

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The protein kinase D (PKD) family occupies a unique position in the signal transduction pathways initiated by B cell receptor (BCR) in normal and malignant cells. BCR engagement initiates a cascade of intracellular events that are crucial for the survival and proliferation of B cells, and may contribute to lymphoma pathogenesis. Using bisulfite sequencing analysis of *PKD1* promoter region we detected methylation at CpG islands that is responsible for PKD1 silencing in B cells. BCR engagement on normal and malignant B lymphocytes induces rapid trans- and autophosphorylation of PKD2. Activated PKD2 was translocated to the nucleus after BCR crosslinking. Moreover, PKD2 was also initiated via CD40 and CD150. We detected differential expression and autophosphorylation of PKD2 in subpopulations of normal B lymphocytes. In plasma cells the level of PKD2 expression was considerably lower in comparison with practically equal high level of PKD2 expression in all other B cell subsets. The highest level of PKD2 autophosphorylation was in germinal center cells. Our data indicate that PKD2 is non-active in resting tonsillar naive B lymphocytes, and becomes rapidly trans- and autophosphorylated in response to BCR engagement during B cell differentiation. Using immunohistochemical approach we demonstrated PKD2 expression and autophosphorylation in the tissue sections of tonsils and different histological variants of B-cell lymphomas. We found diverse PKD2 intracellular localization and autophosphorylation in distinct zones of tonsils: the high level of pPKD2 was detected in both cytoplasm and nucleus of germinal center cells, however, mantle zone cells showed low level of pPKD2 mainly in cytoplasm. We revealed the heterogeneity of non-Hodgkin’s lymphomas by PKD2 expression, intracellular localization and autophosphorylation. According to PKD2 intracellular localization and autophosphorylation diffuse large B cell lymphoma samples were subdivided in three groups.
that also differ by expression of PKCβII, IRF4 and Bcl6. Evaluation of PKD2 expression and its activity in lymphoid malignancies, which represent lymphocytes on different stages of maturation, will help to clarify the role of this enzyme in signaling cascades in normal and tumor B cells.

**SW05.S24–8**
**Nucleocapsid proteins of human paramyxoviruses: antigenic similarities and differences**

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**Background:** Measles virus (MeV) and human parainfluenza virus type 3 (hPIV3) represent respiratory tract pathogens and both belong to *Paramyxoviridae* family. Recombinant nucleocapsid (N) proteins of these viruses form nucleocapsid-like particles (NLPs) that elicit a strong long-lasting humoral immune response [1,2]. Recombinant NLPs can be used to investigate the immunological and biological properties of viral N proteins as well as to detect specific antibodies in human serum specimens. The data on B cell epitopes which would indicate antigenic structure of paramyxovirus N proteins is still limited. The objective of this study was to investigate antigenic structure of these N proteins using monoclonal and polyclonal antibodies (MAbs and PAbs) that were raised in mice against recombinant yeast-expressed N proteins.

**Results:** We have produced recombinant N proteins of different viruses in yeast expression systems. As demonstrated by electron microscopy, recombinant N proteins of MeV and hPIV3 self-assembled to NLPs. To investigate the antigenic properties of recombinant NLPs, we have used panels of PAbs and MAbs raised against NLPs as well as human serum specimens from virus-infected individuals. Antigenic sites recognized by the MAbs were mapped using recombinant overlapping N protein fragments. The major immunodominant sites of MeV and hPIV3 N proteins were localized within their C-terminal region. Further analysis with smaller N protein fragments and overlapping synthetic peptides revealed linear epitopes that were reactive with serum antibodies induced by natural virus infections.

**Conclusions:** The C-terminal regions of paramyxovirus N proteins represent major immunodominant sites, which indicate their surface localization and accessibility to the B cells. These results suggest that the above antigenic sites on the N proteins of human paramyxoviruses are important in eliciting humoral immune response.

**References**


**SW05.S24–9**
**Regulation of CD20 levels in B-cell tumors by SRC family kinases**


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Anti-CD20 monoclonal antibodies have made a breakthrough in the treatment of non-Hodgkin’s lymphoma and chronic lymphocytic leukemia. They trigger indirect effector mechanisms of the immune system, namely complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and immunophagocytosis.

Although for many years CD20 has been described as a stable antigen, accumulating evidence indicates that CD20 can be modulated at both transcriptional and posttranscriptional levels. Downregulation of surface CD20 levels has been linked with tumor resistance to rituximab. Src family kinases (SFKs) including Lyn, Fyn and Lck have been already reported to associate with CD20. However, to the best of our knowledge, the role of SFKs in the regulation of CD20 expression has not been studied so far.

**SW05.S24–10**
**Functional and protective activity of dendritic cells exosomes**

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Exosomes are 40–100-nm diameter membrane vesicles of endoecytic origin that are released by most cell types upon fusion of multivesicular bodies with the plasma membrane, presumably as a vehicle for cell-free intercellular communication.

Functional features and protective activity of exosomes – DC derivatives generated from mice bone marrow have been studied. Exosomes, received from DC, as well as the DC, loaded with a flu virus antigens possessed high functional activity.

In order to study the composition and structure of dendritic cell exosomes we have use methods of proteomic analysis (2D-electrophoresis, MALDI-TOF analysis). Based on proteomic data we can conclude that the composition of these microvesicles is basic proteins of main exosomal complex and fragments of the influenza virus proteins.

Dendritic cells derived exosomes stimulated production of proinflammatory (TNFα, IL-6, IL-1β) and regulatory cytokines (INFγ, IL-12).

Activation of innate mechanisms and representation a virus-specific antigens (H5N2) in structure of exosomes for T-lymphocytes led to formation of adaptive immunity – to occurrence by a rack of an antigen-specific protection against of the flu virus in mice.
**SW06 General Aspects of Biochemistry**

**SW06.S25 Proteomics and Peptidomics (VI-S25)**

**SW06.S25-1 Proteomic and functional comparison of phagocytosis by Fc versus oxLDL receptors in human macrophages**


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The inflammatory activation of macrophages by solid aggregates of oxidized low density lipoprotein (oxLDL) in the intima of the arteries is a decisive event on the path to coronary artery disease. However, the receptor mechanism by which oxLDL aggregates interact with the macrophages to trigger their phagocytosis is not well understood. The role of the receptor signaling complexes in phagocytosis was examined using polystyrene microbeads opsonized with the ligands oxLDL versus immunoglobulin G (IgG). The phagocytosis of 2 μm polystyrene beads opsonized with oxLDL showed different temporal patterns of internalization and modification by serum factors when compared to that of IgG-coated beads. Actin was observed to polymerize at the site of the ligand-microbead receptors and to form a dense layer that surrounded the particles from the time of binding to engulfment. Liquid chromatography and tandem mass spectrometry were used to characterize the receptor complexes associated with oxLDL- versus IgG-coated beads leading to the identification of a detailed list of actin related proteins, binding factors, and signaling enzymes that were specific to the oxLDL- versus IgG-ligand. In agreement with the mass spectral results, treatment with pharmacological inhibitors of SRC, PLC and IAK or the actin cytoskeleton prevented the phagocytosis of both oxLDL and IgG microbeads as measured by laser confocal microscopy. Specific isoforms of PI3K, FAK and SYK were strongly associated with the oxLDL receptor complex and the phagocytosis of oxLDL microbeads was more sensitive to inhibition of PI3K, FAK and SYK activity compared to that of IgG. Silencing RNA against SYK inhibited the phagocytosis of oxLDL microbeads but did not inhibit clearance of IgG opsonized beads. The results showed the differential mass spectral analysis of functionally similar receptors and revealed receptor-specific drug targets that were inhibited or silenced to prevent the inflammatory accumulation of oxLDL particles but that did not prevent bacterial clearance by macrophages.

**SW06.S25-2 Comprehensive, comparative exploration of the Mycobacterium tuberculosis proteome to identify novel vaccine targets, drug targets and disease-associated biomarkers**

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Tuberculosis (TB) is the leading infectious killer of adults worldwide. Since HIV predisposes to tuberculosis disease and up to 1/3rd of people in certain townships are HIV positive, in South Africa today tuberculosis disease is responsible for ca. Twenty percent of all deaths. However, despite over a century of research on the causative organism, *Mycobacterium tuberculosis* (*M. tb*), our ability to rapidly and accurately diagnose and then effectively treat tuberculous infections remains limited, particularly in resource-poor settings. Notably, smear microscopy, which was developed in 1882, has a sensitivity of only ~65%, yet is still the gold standard test for TB diagnosis in the field in the enveloping world. The advent of nucleic acid-based tests such as the GeneXpert are revolutionising the diagnosis and and drug-resistance typing of TB suspects in well resourced settings, but there remains a pressing need for improved point-of-care diagnostic tests that are rapid, accurate, cheap, require no external power and which can therefore be used in resource-poor settings. In addition, new drug- and vaccine targets and drug candidates, as well as an improved understanding of the evolution of drug resistance in *M. tb*, are urgently needed to combat the alarming emergence of multi-extensively and totally-drug resistant *M. tb* strains. New biomarkers of TB disease, of *M. tb* phenotype, and of the response of *M. tb* to drug treatment are thus desperately needed today.

We have carried out a comprehensive cross-species proteomic analysis of eight pathogenic and non-pathogenic mycobacteria and have quantified ~3300 protein in each organism, representing ca. 80% of each theoretical proteome, enabling proteogenomic re-annotation of the individual organisms. Through cross-correlation of our proteomic data, including with gene expression models of *M. tb* cultured under varying experimental conditions, we have identified candidate biomarkers that may correlate with the clinical phenotypes of virulence and pathogenicity and which may therefore represent plausible vaccine targets. Based on our comprehensive mycobacterial proteome coverage, we have used MRM assays to quantify the response of the *M. tb* proteome on exposure to sub-MIC concentrations of a drug with anti-mycobacterial activity, 5-fluorouracil, which has confirmed a plausible dual targeting mechanism for this drug in *M. tb* and may therefore aid the development of novel anti-tubercular compounds. In addition, building on our mycobacterial proteomic research, we have identified a set of novel mycobacterial proteins present in urine samples from TB patients that represent candidate diagnostic markers of disease.

**SW06.S25-3 The human nucleolar protein SURF6 affects degradation of the pre-rRNA internal transcribed spacers and interacts with a number of rRNA processing factors**

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Mammalian SURF6 (in human – hSURF6, 361 a.a., PI 10.51) is a specific nucleolar protein that is absolutely necessary to maintain cell viability. However, SURF6 roles in mammalian cells have not been studied intensely and still remain uncertain.

To shed some light on a role of hSURF6 in ribosome biogenesis, we examined the effects of the hSURF6 level on the levels of the major structural domains of the 47S rRNA precursor (pre-rRNA), including 5′ETS, 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, by quantitative real-time PCR (qRT-PCR).
HeLa cells were transfected either by a plasmid, which assures ectopic overexpression of hSURF6, or by a plasmid which provides for hSURF6 mRNA post-transcriptional knockdown. Our results showed that SURF6 overexpression drives to accumulation of the pre-rRNA internal transcribed spacers ITS1 and ITS2, whereas depletion of the hSURF6 pull leads to exhaustion of their levels. The content of other pre-rRNA domains was not significantly affected. These observations for the first time showed that hSURF6, like as its yeast homologue the protein RRP14/yl082c, is involved in stabilization (or prevention of degradation) of pre-rRNA. Neither protein with a similar function has so far been described in the human proteome.

To examine whether hSURF6 interacts with any rRNA processing factors, we applied the GST pull-down assay and co-immunoprecipitation assays. The proteins co-interacted with hSURF6 were identified in HeLa cells nuclear extracts by mass-spectrometry and, in independent experiments, verified by Western blot analysis with specific antibodies. We demonstrate that hSURF6 forms an RNA-independent complex with a number of rRNA processing factors, including nucleophosmin/B23, Nop52 and EBP2, but does not interact with ISG20L2 – an exonuclease involved in 5.8S rRNA maturation. At the cellular level SURF6 is colocalized with nucleophosmin, Nop52 and EBP2, as well as with ITS1 and ITS2.

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**Abstracts**

Human serum metallomics and proteomics

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We used metal affinity chromatography to enrich a fraction of human serum proteins on immobilized columns loaded with cadmium, nickel, zinc, copper, or lead in bis Tris saline and these proteins were identified using LC-MS/MS. Tens of enriched proteins were identified and we present here the 20 most abundant proteins, including ASNS and mPR, involved in various drug-resistance-forming mechanisms. Our results provide useful diagnostic markers and therapeutic candidates for the treatment of doxorubicin-resistant uterine cancer.

**References**


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**Human serum metallomics and proteomics**

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Drug resistance is a common cause of failure in cancer chemotherapy treatments. In this study, we used a pair of uterine sarcoma cancer lines, MES-SA, and the doxorubicin-resistant MES-SA/Dx5 as a model system to examine resistance-dependent cellular responses and to identify potential therapeutic targets. We used two-dimensional differential gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) to examine the global protein expression changes induced by doxorubicin treatment and doxorubicin resistance. A proteomic study revealed that doxorubicin-exposure altered the expression of 87 proteins in MES-SA cells, while no significant response occurred in similarly treated MES-SA/Dx5 cells, associating these proteins with drug specific resistance. By contrast, 37 proteins showed differential expression between MES-SA and MES-SA/Dx5, indicating baseline resistance. Further studies have used RNA interference, cell viability analysis, and analysis of apoptosis against asparagine synthetase (ASNS) and membrane-associated progesterone receptor component 1 (mPR) proteins, to monitor and evaluate their potency on the formation of doxorubicin resistance. The proteomic approach allowed us to identify numerous proteins, including ASNS and mPR, involved in various drug-resistance-forming mechanisms. Our results provide useful diagnostic markers and therapeutic candidates for the treatment of doxorubicin-resistant uterine cancer.

**Abstracts**

**Gaining insights into the role of tumor initiating cells in colon cancer by a multitask approach**


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A small subset of tumor initiating cells (TICs) has been revealed in several cancers. In colon cancer, these cells were identified on the basis of CD133 expression but, despite recent progresses, not many strategies for TICs purification are available and data about TICs pathways are emerging only slowly. In this study, we explored colon TICs ‘outside’ and ‘inside’ by using two different approaches in the attempt to achieve the broadest possible understanding of the outcome. First we carried out a phenotypic characterization of colon TICs (isolated from CaCo-2 using CD133 expression as reference) by flow-cytometry founding that CD66c expression was brightest in CD133+ cells, while in CD133− cells. Importantly, analyzing fresh tissues we confirmed that CD66c expression was specifically associated with TICs and finally, in vitro and in vivo experiments demonstrated that CD66c silencing almost completely abrogated the tumorigenic potential of CaCo-2 cells. In the mean time, we analyzed by a proteomic gel-based approach (2D-DIGE), the protein expression profile typical of TICs isolated from CaCo-2 and HCT-116. Among the proteins differentially expressed in TICs, we found a 2-fold change up-regulation of the splicing factor SRp20, a target gene of the Wnt/β-catenin pathway. Moreover, we revealed a direct cause-effect relationship between Wnt pathway activation and the SRp20 expression. We showed that SRp20 influences cell proliferation thereby implicating SRp20 in the tumorigenicity of TICs and our results demonstrated that when SRp20 is silenced, the expression of the negative regulator of the Wnt pathway MCC is increased, while β-catenin is decreased, which suggests a slowing-down of the Wnt pathway. These data suggest that SRp20 could modulate the Wnt pathway by affecting MCC expression. In conclusion, our results open the way for a possible role of CD66c and SRp20 in the TICs-targeted therapy.

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**References**

tions have also been identified. The metal array use in the proteomic biomarker search technologies gives this data particular importance. In addition, this data compliments our studies of the effects of lead and mercury on the serum proteome in children.

**SW06.S25–7**

A proteomic approach to study malignant pleural mesothelioma

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Malignant pleural mesothelioma (MPM) is a rare and devastating primary tumor of the pleura. Its prognosis is very poor, the median survival after diagnosis is <12 months. The predominant cause of MPM is the asbestos exposure, and, even if many efforts have been made to remove asbestos from workplace, new cases are continually diagnosed seeing the long latency after asbestos exposure. An early diagnosis could improve outcomes but at present there are not specific and sensitive markers. The aim of this study was to compare proteome profile of epithelioid mesothelioma and benign biopsies, using a proteomic approach. Tissue biopsies were obtained from 53 patients who were subjected to a diagnostic thoracoscopy. After histological examination, 22 samples were classified as epithelioid MPM, 20 as lung carcinoma and 11 as benign. Benign samples were used as negative control while lung carcinomas were used in the validation step as positive control. Samples were submitted to two-dimensional electrophoresis, stained with Sypro, and images were analyzed with Progenesis Same Spot software performing a comparison between MPM and benign. Thirty-seven proteins that showed a fold variation ≥3 were identified by NanoLC-ESI-MS/MS analysis. Moreover, the differential expression of proteins of interest has been validated by western blot analysis. We confirmed known potential biomarkers, and suggested new proteins. In particular, interesting values of sensitivity and specificity for pre-lamin A/C, vimentin, and calretinin were obtained. Finally, Ingenuity Software was used to investigate the relationship between these proteins and various biological pathways. Overall our results propose a specific panel of proteins that are able to distinguish MPM, and that could add information to understand biological characteristics of MPM.

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**SW06.S25–8**

Comparable effect of different heavy metal ions on *Enterococcus hirae* membrane vesicles ATPase activity

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Small amounts of some of heavy metals, such as Cu and Fe, are essential and required by microorganisms for a number of essential enzymes [1,2]. It is known that the growth and membrane activity of different bacteria are markedly affected by different heavy metal ions with oxidative and reducing properties [1,2]. It was suggested that the targets of heavy metal ions effects in bacterial cells might be the membrane-associated ATPases [3].

In order to examine the role of the membrane enzyme in heavy metal effects, the ATPase activity of *E. hirae* wild-type ATCC9790 membrane vesicles were investigated in the presence of Fe and Cu ions (within the range of 0.05–1 mM) with or without N,N′-dicyclohexylcarbodiimide (DCCD), inhibitor of the FoF₁-ATPase. It was shown that these ions directly affect the FoF₁-ATPase. In all cases (DCCD+/-) even low Cu²⁺ concentrations decreased the overall ATPase activity by a concentration dependent manner. Similar results were obtained with Fe³⁺. These results can be explained by direct action of these ions on FoF₁ modifying its activity and interaction with secondary transport systems. Meanwhile in the presence of other oxidizer heavy metal ions, Fe³⁺, a higher ATPase activity was observed in comparison with control even together with 0.1 mM DCCD. These effects may be explained by existence of Fe³⁺-dependent ATPase in *E. hirae*, different from FoF₁, that activity can be observed even when FoF₁ is inhibited by DCCD. To reveal specific effects of these metal ions, ATPase activity was investigated in the presence of the other divalent heavy metal ions, Ni²⁺ and Cu²⁺. It was shown that these ions had no effects on ATPase activity.

Taken together, the results indicate that in case of heavy metal ions mentioned above special action mechanisms can be evaluated. These ions directly affect membrane ATPase and their effects do not depend on oxidative and reducing properties of heavy metals.

**References**


**SW06.S25–9**

Mesopore-assisted fingerprints of gingival crevicular fluid by MALDI-TOF mass spectrometry for monitoring inflammatory state in patients wearing fixed orthodontic appliance

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Gingival crevicular fluid (GCF) is an exudate secreted by the gums that can be found in the crevices located at the point where the gum line meets the teeth. The physiological amount of this fluid is usually limited, but it can increase when an inflammatory process takes place in the mouth. Therefore, GCF is a convenient, noninvasive and efficient means to sample biomarkers of inflammation and bone resorption in the oral cavity. However, only few studies so far investigated the role played by the GCF peptidomic components in inflammatory conditions and other physiopathological processes that occur in the oral cavity. In the research area of clinical proteomics, peptidome mass spectrometry-profiling of human bodily fluids represents a promising tool for novel biomarker discovery [1,2,3]. Based on a molecular cut-
off mechanism, we used mesoporous silica particles (MSP) as sponges to ‘capture’ peptides present in human plasma, urine, induced sputum by excluding large size and high abundant proteins from adsorptive process. We are now revisiting our strategy based on MSP for rapidly profiling GCF. MSP with different pore diameter ranging from 2 to 8 nm and with different surface chemistry were tested on GCF. In particular, SBA-15 derivatized MSP with pore size of about 6 nm showed the best results in term of peptide extraction exhibiting the highest number of peaks with an high S/N ratio. Additionally, preliminary data on intra-day and interday MALDI-TOF spectral profiles showed no significant differences. As a proof-of-principle, we challenged our platform in the identification of a panel of biomarkers which are associated with the extent of gum inflammation. Preliminary results indicate that several m/z peaks from GCF peptidome emerged as potential diagnostic peptidic patterns which correlate with the gums inflammatory state in patients wearing fixed orthodontic appliance. These findings may contribute in the definition of a MS-based platform for high-throughput screening identification of peptide-biomarkers of gums inflammation.

References

SW06.S25–10
A proteomic view on factors associated with high virulence of Staphylococcus aureus in chicken embryo model
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Staphylococcus aureus, a major threatening opportunistic pathogen, is a leading cause of numerous infections and diseases of its humans and animals hosts. The ability of the bacterium to effective host colonization and disease development is determined by the production of a range of virulence factors secreted into extracellular milieu. Previous studies revealed clear differences in the virulence of poultry-associated S. aureus strains tested using a chicken embryo but not nematode model and their correlation with the strain genotype [1]. This suggests the existence of host-specific virulence factors. The aim of the study was to verify whether the differences in virulence are also reflected at the proteomic level, and to identify proteins that potentially acts as virulence factors.

Series of comparisons of extracellular protein profiles of well characterized poultry-originated, S. aureus strains, previously identified as virulent (seven strains) and non-virulent (five strains) were conducted. Proteins from culture fluids were precipitated, labeled with fluorescent dyes and subjected to two-dimensional difference gel electrophoresis in pairs (virulent vs non-virulent). Differentiating spots, reflecting proteins present-only or exhibiting higher abundance in the culture fluid from the virulent strain, were cut out from the gel, trypsin digested and subjected to mass spectrometry (MS) analysis.

In the course of fifteen conducted comparisons 325 differentiating spots exhibiting higher intensity in virulent strains were identified. The majority of the differences were qualitative. During MS analysis 122 different proteins were identified. Two of those proteins (bifunctional autolysin and alpha-hemolysin) appeared in all virulent examined strains, another two (1-phosphatidylinositol phosphodiesterase and staphopain B) in six strains, four were present in five and eight in four virulent strains, respectively. We speculate that these proteins might play a significant role as poultry-host-specific virulence factors.

The obtained results expand the knowledge on protein effectors of staphylococcal virulence and constitute the step forward in our understanding the process of host-specificity of S. aureus strains.

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Reference

SW06.S25–11
Ultra-miniaturisation of tandem affinity purifications: a new frontier in dissecting/mapping organelar interactomes
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Tandem affinity purification (TAP) coupled to high-end, modern mass spectrometers has proven to be a robust method for the isolation and analysis of non-covalently interacting protein complexes [1,2]. The combination of a streptavidin (Strep)-binding peptide and a haemagglutinin (HA) motif enables a two-step enrichment protocol that can effectively capture the tagged protein and interacting partners. Further improvements such as chemical crosslinking to irreversibly attach transiently-binding partners in the complex now allow a more deep and thorough analysis of protein complexes. Together these methods have advanced the analysis of cellular interactomes. Currently, however, such approaches still require a large amount of starting material and thus in some regard, limit the technique.

Here we propose the use of a modified TAP approach that opens the possibility of reducing the quantity of input protein, whilst maintaining the same level of protein identification as with larger, standard quantities. Using a smaller column bed volume with proportionally-scaled volumes of beads, we have achieved similar results to that obtained with a full-sized TAP pulldowns. We have verified this approach by the use of both a C-terminally linked Strep-HA-green florescent protein (GFP) and an N-terminally linked Strep-HA-TANK-binding protein 1 (TBK1) expressed in HEK293 Flp-In tetracycline inducible cells. To minimise protein loss, a one-step TAP was performed using Streptactin beads and elution with biotin, followed by tryptic digest and LC-MS on an Agilent 1200 series HPLC coupled to a Thermo Fisher LTQ Orbitrap Velos. Raw MS data were matched to peptides and proteins using MASCOT and Phenyx with a false positive detection rate at the protein level of 1%. This new miniaturisation of the TAP can be applied to the elucidation of organelar complexes. Thus a robust and exacting technique such as this has the potential to determine protein networks in organelles, for example the ER, mitochondria and peroxisome, that have until now been poorly explored.

References
Modulation of cytokine and angiogenic factors on glioblastomas

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Cytokine dysregulation has an important role in a number of cellular processes in cancer. Glioblastomas are associated with a large number of cytokines with deregulated expressions, and are generally associated with increased angiogenesis.

The aim of this study was to evaluate the cytokine profile in gliomas in order to establish a panel of biomarkers useful in early tumor detection.

We determined angiogenic factors and cytokine levels in sera from 33 glioma patients and 30 healthy individuals. Using Milliplex™ MAP Human Cytokine/Chemokine Panel (Millipore, MA, US) on a Luminox® 200™ system, we analyzed 12 analyte-specific bead sets: pro-inflammatory IL-1β, IL-2, IL-6, IL-8, IL-12, TNFα, GM-CSF, INFγ, anti-inflammatory IL-4, IL-10, and angiogenic factors VEGF and FGF-2. Multiplex data acquisition and analysis were performed using STarStation 2.3 (Applied Cytometry Systems, Sheffield, UK).

Multiplex analysis indicated a strong overexpression for some of the pro-inflammatory cytokines – IL-1β, IL-6, and TNF-α and for the anti-inflammatory cytokine IL-10 (over 3-fold stimulation in glioblastoma patients). We also found significant up-regulation (1.1–2 fold) was found a significant up-regulation (1.1–2 fold) for the angiogenic factors VEGF, FGF-2, and the pro-inflammatory cytokines IL-8, IL-2 and GM-CSF. For these cytokines, expression was strongly correlated with tumor grade, proliferation markers and clinical aggressiveness in glioblastomas. For the other analytes results showed no significant differences between the glioma and control groups.

A panel of pro-inflammatory – IL-1β, IL-6, TNF-α, IL-8, IL-2, GM-CSF, anti-inflammatory cytokine IL-10 and angiogenic factors VEGF, FGF-2 are closely linked to the gliomas behavior. sMAP technology might be a suitable tool for evaluation of different molecules involved in tumoral development, among them cytokines and angiogenic factors. Further analysis could generate a panel serving for a better patient stratification and more adequate therapeutic approaches.

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Search of proteins associated with outgrowth of murine metastasis following primary tumor removal

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In clinical practice, surgical removal of primary tumors is often accompanied by an exceptionally rapid outgrowth of distant metastases. This effect is often associated with production of angiogenic inhibitors by the primary tumor. At the same time it was shown that metastasis evolved only from the removed tumor in experimental mice bearing two different types of tumors, and this specificity cannot be mediated by the known angiogenic factors.

We tried to identify specific protein regulators associated with activation of metastasis after the tumor removal using Murine Ehrlich Carcinoma.

30% of Ehrlich ascites tumor is tumor cells. Therefore evacuation of ascitic tumor from the abdominal cavity can be considered the removal of the primary tumor. Eighty percent of Ehrlich ascites was collected from the mice with ascitic Ehrlich carcinoma 10 days after the tumor cells had been injected (control groups). The mice from the 1, 2 and 3 experimental groups were sacrificed 3, 7 and 24 h, respectively, after the primary tumor removal. The abdominal cavities of the mice were dissected and the rest of ascitic fluid was collected.

We carried out the comparative analysis of protein profiles of control and experimental groups. The ascitic fluids from the mice were analyzed using 2D-DIGE and LC-MS/MS analysis separately. The LC-MS/MS analysis was carried out on the ABSciex TripleTOF 5600+.

The samples of the ascitic tumor were subjected to the analysis resulted in identification of more than 400 proteins. The joint results of the two methods gave 21, 24, and 15 identified unique proteins in the 1, 2 and 3 experimental groups, respectively.

Search for rapid and reliable methods of socially significant disease diagnostics is one of the high-priority areas of modern medicine. In consideration of the recent advances, proteomics approaches are expected to give a clue to new biomarkers in human blood plasma and serum. Among proteomics technologies mass-spectrometry is considered to be one of the most sensitive methods for compound screening analysis in biological samples.
duced quantitative analysis by means of Total Ion Current method revealed proteins significantly up- and down-regulated. A number of proteins potentially associated with activation of metastasis after surgical primary tumor removal were identified.

**SW06.S25–15**

**Integrating peptidomic with lipidomic fingerprints by using mesoporous aluminosilicate and MALDI-TOF MS**

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MS technologies offer the opportunity to screen and discovery simultaneously multiple biomarkers, which consist of a pattern of up- or down-regulated molecules (proteins, peptides, lipids, metabolites) representative of a given (healthy/disease) condition [1–3]. Endogenous peptides (PT) and lipids (LP) represent two important classes of biomolecules owing to their multiple biological roles in physiological and in pathological conditions. Considering how deeply protein activity regulates the lipidic pathway, studies of both classes, by complementing each other, can be promising in disease monitoring. As a part of an ongoing project aimed to explore the effectiveness of mesostructured materials in proteomic analysis we have found that mesoporous aluminosilicates (MPAS) show an excellent selectivity for PT and LP [4]. Specifically, MPAS are able to ‘capture’ PT and LP present in human body fluids as a result of a variety of interactions and a cut-off mechanism by which preferentially PT and LP which diffuse into the mesoporous channels (nanometric in size), are selectively adsorbed. Following the extraction, MALDI-TOF MS analysis with the suitable matrix and instrument settings provides the rapid visualization of PT and LP signatures captured by MPAS in a small amount of specimen [4]. Therefore this platform allows the optimal integration of peptidomic with lipidomic profiles which is particularly useful for many trans-omics studies. In the present study we have applied the ‘MPAS-MALDI strategy’ in order to distinguish, osteoarthritis (OA) patients with different degrees of disease severity. Different subsets of OA patients (10 grade I, 6 grade II, 8 grade III, 7 grade IV, 6 grade V) were compared. The differential analysis of peptidomic and lipidomic m/z peaks (more than 150 peaks in MALDI-TOF MS analysis) allowed the identification of 10 peaks (6 peptides and 4 phospholipids) which were differentially expressed in low grade patients compared to high grade patients. These species were assigned by MALDI-TOF/TOF MS analysis.

**References**


**SW06.S25–16**

**Adhesive proteins and crosslinking enzymes are concentrated together on a spot on the surface of the phosphatidylserine-expressing activated platelets**

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Morphogenesis, tissue remodeling, immune and hemostatic responses are dependent on the ability of cells to bind to each other and to the extracellular matrix that is mediated by various adhesive proteins. Additional crosslinking by transglutaminases is often involved in these processes. Previous studies showed that spatial distributions of such proteins can be non-uniform, it is important for their regulation.

Our aim was to study the distribution of adhesive proteins fibrin(ogen), thrombospondin (TSP) and crosslinking enzymes tissue transglutaminase (tTG) and factor XIIIa on the surface of the phosphatidylserine (PS)-expressing activated platelets.

Washed gel-filtered platelets were activated for 15 min with 100 nM thrombin and/or 20 μg/ml collagen-related peptide (CRP) in presence of 2.5 mM CaCl2. Activated platelets were labelled in different combinations by fluorescent markers for PS, fibrin(ogen), TSP, tTG and factor XIIIa. Then they were analyzed by confocal microscopy.

The experiment showed the PS-expressing platelets could be found in free form and among the aggregates. The major adhesive proteins, such as fibrin(ogen), TSP and major crosslinking enzymes tTG, factor XIIIa were detected both on the single PS-expressing platelets and those involved in the platelet aggregates. These proteins were concentrated on a single spot instead of a uniform distribution in all cases. The PS-expressing platelets in such aggregates were connected to the non-PS-expressing ones only through the spot. Such spot was only one per each cell. In case of thrombin or thrombin-plus-CRP platelet activation the fibrin(ogen) spot had higher density compared to CRP activation. PS staining revealed that this structure is membrane-based.

These data suggest a revised model of the PS-expressing platelet adhesion based on the non-uniform protein distribution.

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**SW06.S25–17**

**Comparative analyses of peptidome and proteome of CSF samples from patients with Guillain–Barre syndrome and with non-neurological diseases**

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Guillain–Barre syndrome (GBS) is an immunemediated inflammatory disease of the peripheral nerves, involving both the myelin sheath and the axons. Damage to these structures causes biomarkers to be released into the adjacent body fluid compartment. Our main objective was to clarify the mechanisms of degradation of the myelin sheath of peripheral nerves. We discovered unique protein samples in patients with GBS, which are not found in healthy patients. The resulting list of peptides and proteins contained in the cerebrospinal fluid (CSF), compare it with the list of peptides and proteins contained in serum samples.

The patient group consisted of 13 patients with GBS. The GBS group included patients with acute inflammatory demyelinating polyneuropathy (AIDP). The control group consisted of 20 patients with non-neurological disorders with normal CSF examination results. CSF peptidome was done by reverse-phase chromatography and LC-MS/MS. Using 1D-electrophoresis and LC-MS/MS techniques, we aimed to investigate the CSF proteome. The analysis was carried out on ABSciex TripleTOF 5600+.

After the analysis and identification peptides and proteome of the control group patients, we identified 775 of the 120 peptide precursor proteins in peptidome and 408 proteins in the proteome. After receiving the results of the identification peptidome we found 754 of the 110 peptide precursor proteins from patients
with GBS. We compared the results and found that the 314 peptides and 13 precursor proteins are unique to patients with GBS and were not found in control samples. Among all the identified proteins was not identified Myelin basic protein (MBP). This result is very interesting for further study of GBS.

In the following experiments, we want to clarify the mechanisms of degradation of the myelin sheath of peripheral nerves and use SWATH method for quantitative comparison.

SW06.S25–18
Production of hen egg IgY liposomes against different salmonella species
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Hen egg contains immunoglobulins (IgY), whose function is to protect chickens against harmful microorganisms such as bacteria, viruses and fungi. IgY is predominant in the immunoglobulin fraction of sera of birds, reptiles and amphibians. Immunoglobulins are transferred from the plasma of dam into the egg yolk through a IgY receptor found in the yolk sac thus providing passive immunity to the offspring. Antibodies extracted from egg yolks have been used as an alternative to mammalian immunoglobulins, since IgY represent the avian counterpart of IgG. The objective of our study was to isolate and estimate the in vitro activity of egg yolk IgY against different Salmonella species. So we created four groups of twenty hens that were immunized two times with different salmonella vaccines and with alum adjuvant. Egg yolk was separated from egg white and specific IgY with high purity was isolated from yolks by water dilution, salt precipitation and ultrafiltration. Enzyme-linked immunosorbent assay (ELISA) indicated the isolated IgY specifically targeted Salmonella. Activity of the antibody was tested using commercial ELISA, Western blotting, and in vitro microbiological assays. In the second part of our study, we prepared IgY liposomes that had been capsulated with lipids extracted from egg yolk and determined the activities of these IgY liposomes. Our results showed that egg yolk may be used for large scale production of specific antibodies against Salmonella. Since hen egg is a cheap and convenient source of specific antibody production, further applications of the IgY liposomes should be possible for treatment and prevention of various infections in both animals and humans.

SW06.S25–19
Formation of the complex between human thymidylate synthase and dihydrofolate reductase, the enzymes involved in thymidylate biosynthesis
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Thymidylate synthase (TS) catalyzes the sole de novo thymidine monophosphate (dTMP) synthesis, a process necessary for DNA synthesis and repair, which determines cell proliferation. The enzyme, together with dihydrofolate reductase (DHFR) and serine hydroxymethyl transferase is an element of thymidylate synthesis cycle. As the deficiency of TS or DHFR leads to cell death, both enzymes are highly important drug targets in many different pathology disorders (e.g. cancer; autoimmune diseases; infections: malaria, pneumonia, toxoplasmosis). DHFR inhibitors are used extensively in the therapy of such diseases as leukemia, lymphoma and choriocarcinoma, while TS inhibitors are primary drugs in chemotherapy of the gastrointestinal tract, head and neck cancers.

In majority of organisms, including humans, TS and DHFR are separate enzymes. TS amino acid sequence is one of the most conserved among known proteins (over 90% of homology among mammals and over 80% of homology between mammals and bacteria). On the other hand, DHFR has homologous sequence among vertebrate species (75–95% of homology), while homology of vertebrates DHFR sequence to lower eukaryotes or bacteria DHFR sequence is about 30%. Conversely, in protozoa and plants DHFR and TS are encoded by a single gene and expressed as one bifunctional DHFR-TS polypeptide chain. Domains are located on the opposite ends of the chain, have autonomous binding sites and reveal the same properties as monofunctional proteins. Importantly for our studies, it has been proved that domains interact with each other in native high-order structure of DHFR-TS enzyme and mere physical presence of DHFR is necessary for biological activity of TS. Moreover, a substrate kinetic ‘channeling’ between active sites of the domains has been observed.

Considering the possibility of future designing drugs targeting the TS-DHFR complex instead of individual enzymes, we undertook the studies aiming at thorough understanding of mutual interaction between human TS and DHFR. Our studies involving native electrophoresis, Western and Far-Western experiments have shown the complex formation between these two enzymes. Furthermore, the binding of TS with DHFR has been characterized by various methods, including microscale thermophoresis and quartz crystal microbalance. In addition, influence of posttranslational modifications such as TS phosphorylation on the protein complex dissociation constant has been observed. Last but not least, we have conducted the bioinformatics projection of the complex hDHFR – hTS based on available crystallography structures of bifunctional DHFR-TS proteins.

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SW06.S25–20
QCM-D study of the serine hydroxymethylotransferase – thymidylate synthase – dihydrofolate reductase tri-complex
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Serine hydroxymethylotransferase (SHMT), thymidylate synthase (TS) and dihydrofolate reductase (DHFR) play a key role in the process of the sole de novo thymidylate biosynthesis, which is essential for DNA replication and genome stability in the nucleus and mitochondria. DHFR reduces dihydrofolate to tetrahydrofolate using NADPH as a cofactor, TS methylates 5-deoxyuridine monophosphate forming thymidine monophosphate, whereas SHMT transfers a methylene group from serine to tetrahydrofolate. Enzymes of the thymidylate biosynthesis cycle are high potential drug targets, as their deficiency leads to the ‘thymineless death’ of a cell. Previous studies have shown that SHMT plays a crucial role in the folate cycle not only by its catalytic activity. It also serves as a scaffold protein that is essential for the assembly
of the de novo thymidylate synthesis pathway into a multienzyme complex in the nucleus. Therefore, presence of SHMT is required for co-localization of DHFR and TS to the nuclear lamina.

The aim of our study was to investigate the mechanism and kinetics of the human multienzyme SHMT-TS-DHFR complex formation in order to fully characterize and understand the interactions occurring between the enzymes that constitute the de novo thymidylate pathway. In this study quartz crystal microbalance with dissipation mode (QCM-D) was used to investigate the process of the protein tri-complex formation. QCM-D is an ultra sensitive technique based on the piezoelectric phenomenon of quartz. It allows the full quantitative analysis and kinetics determination of the binding process occurring on the surface of a quartz crystal sensor. The studies showed that neither TS or DHFR specifically bind to SHMT alone. The complex is formed only in presence of both – thymidylate synthase and dihydrofolate reductase. The obtained data have been furthermore confirmed by means of microscale thermophoresis (MST).

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**SW06.S25–21**

A central fragment of ribosomal protein S26 containing the eukaryote-specific motif


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One of crucial ribosomal ligands is messenger RNA (mRNA) bearing the genetic information. During translation, the ribosome binds a 30–50 nucleotide mRNA fragment including codons interacting with tRNAs. Site-directed cross-linking studies with application of different mRNA analogs showed that ribosomal protein S26 (rpS26e), which has no eubacterial counterparts, is the main component of binding site of the mRNA region 5′ of the codons interacting with tRNAs [1].

Here, the rpS26e oligopeptide neighbouring mRNA on the human 80S ribosome was determined. rpS26e was cross-linked to 5′-32P-labeled mRNA analogs bearing Phe codon UUU at the 3′-end and a 4-azido-2,3,5,6-tetrafluorobenzoyl cross-linker at an U residue at the 5′-end. Cross-linking was carried out in specific human 80S ribosomal complexes obtained in the presence of rNAPhe that directed triplet UUU to the ribosomal P site and positioned the modified mRNA nucleotides in designed locations 5′ of the E site codon. Identification of cross-linking sites on the rpS26e was carried out using an approach based on the application of various proteolytic agents for selective cleavage of the cross-linked protein with subsequent SDS-PAGE separation of the labelled modified peptides, their identification and mapping of the cross-links. In all complexes, cross-linking occurred to the same dodcapeptide in positions 60–71 in the central part of the protein. An analysis of protein sequences of the rpS26e family revealed that motif YxxPKxYxK within the mentioned fragment is conserved in eukaryotes but not in archaea. A comparison of the model of the 40S subunit [2] with available cryo-EM images of the 40S subunit complexed with eIF3 [3] shows that the location of rpS26e on the 40S subunit overlaps with the eIF3 binding site. It was suggested that this motif interacts with eIF3, which is involved in recruitment of the mRNA to the 40S subunit and has no counterparts in bacteria and archaea.

The work was supported by the Presidium of Russian Academy of Sciences (Program on Molecular and Cell Biology, grant to G.K.) and by the Russian Foundation for Basic Research (11-04-00597 to G. K.).

**References**


**SW06.S25–22**

Proteomic analysis of exosomes secreted by human glioblastoma cells hold promise for identifying markers of brain cancer

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Exosomes are endosomally derived 20–100 nm membranous vesicles secreted by many cell types into the extracellular compartment. Exosomes contain a characteristic composition of proteins, mRNAs and microRNAs which can be transferred to recipient cells. Exosomes have been studied for their ability to function in intercellular communication and are promising area for biomarker discovery.

In the current study, the exosomes of five human glioblastoma cell lines were examined *in vitro*. Characterization of these small particles remains a challenge. Here these vesicles have been characterized further by mass spectrometry, one-dimensional (SDS-PAGE) and two-dimensional electrophoresis (2DE), immunoblotting, atomic force microscopy, and light-scattering methods. The current study employed several original approaches, as well as standard methods such as series of centrifugation steps with ultracentrifugation to isolate exosomes from the cultural media. By using these methods, we isolated exosomes that was confirmed by dot-blot analysis for various exosomal proteins including CD63 and HLA-ABC. Additionally, we demonstrated that exosomal preparation was devoid of such major protein as bovine serum albumin (BSA), which is often a contaminating factor from cultural medium suggesting the presence of other non-exosomal proteins in the sample.

SDS-PAGE analysis of proteins obtained from exosomes of glial cells and noncancerous cells showed difference in protein profiles. Proteins from exosomes of glioblastoma cell lines were separated by two-dimensional electrophoresis (2DE). Protein profiles were further analyzed by densitometry and mass-spectrometry (MS) that allowed identifying more than 30 proteins. Most exosomal proteins are related to endocytic compartments. Other exosomal residents include cytosolic proteins most likely involved in exosomes biogenesis and function (cofilin, hsp70, hsp90) and intracellular membrane transport and signaling (such as annexins). Our findings show that exosomes from glioblastoma cells have proteomic profiles similar to vesicles from other cell types and to the cells of their origin. Furthermore, we also identified by MS and Western blot analysis a novel category of exosomal proteins related to apoptosis: PCNA and p53. These proteins were presented in five glial exosome samples but were not in exosomes from normal non-cancerous cells. This distribution is well correlated with the presence of these proteins inside of cells themselves. Overall, this study suggest the panel of specific brain tumor exosomal markers to help create noninvasive techniques to diagnose disease.
**SW06.S25–23**

**Actin-binding protein alpha-actinin 4 (ACTN4) is a transcriptional co-activator of RelA/p65 sub-unit of NF-kB**

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Alpha-actinin 4 (ACTN4) is an actin-binding protein which belongs to the spectrin superfamily. It predominantly localizes to the cytoplasm and focal adhesion contacts. Primarily, ACTN4 was found to participate in the cytoskeleton organization and cytokinesis, regulating cell adhesion and shape. Being a part of cytoskeleton, ACTN4 plays a crucial role in remodeling of actin cytoskeleton, and in formation of protrusions, which potentiate migration of normal and cancer cells. ACTN4 resides both in the cytoplasm and nucleus where it can physically associate with various transcription factors. Hereby, we describe an effect of ACTN4 expression on transcriptional activity of the RelA/p65 subunit of NF-kB. We demonstrate that ACTN4 enhances RelA/p65-dependent expression of c-fos, MMP-3 and MMP-1 genes, but it does not affect TNC, ICAM1 and FN1 expression. Importantly, actin-binding domains and dimerisation of ACTN4 are not critical for the nuclear translocation and co-activation of RelA/p65-dependent transcription. Collectively, our data suggest that in the nucleus, ACTN4 functions as a selective transcriptional co-activator of RelA/p65.

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**SW06.S25–24**

**A group of brain exo- and endo-metalloproteases bound to axonal ends of neurons (NEMPs); some specific properties, ways to protect therapeutic peptides**

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For communications in brain, nerve cells besides synaptic contacts actively use the intercellular medium. Neurotransmitters secreted by cells or introduced in this medium regulate cellular metabolism by affecting the nerve cell receptors. A number of extracellular proteases control dynamics of neurotransmitters. Matrix metalloproteases regulate the state of extracellular matrix made mainly of collagen fibrils. The progress of axonal ends of neurons (growth cones) through matrix towards the target cells depends on matrix metalloproteases activity.

We have discovered that a group of peptidases is bound to the outside of plasma membrane of neurons: Four exopeptidases were detached from isolated axonal ends (synaptosomes) by 0.1% Triton X-100. Activities of all these neuron bound peptidases are inhibited by 1,10-phenanthroline. It means that these enzymes are metallopeptidases. They were named Neuronal Ecto MetalloPepidases (NEMPs). The peptidases were characterized as:

1. Carboxypeptidase (NEMP1)
2. Aminopeptidase (NEMP2)
3. Endopeptidase A (NEMP3)
4. Endopeptidase B (NEMP4)

Both exopeptidases (NEMPs 1and 2) can split dipeptides, but not carnosine. Therefore carnosine placed on C-end, on N-end or on both ends of a peptide protects from NEMP1, from NEMP2 or from both, correspondingly. Instead of carnosine its constituent β-alanine could be used with the same result. NEMP1 is composed of penetrating through pores of dialysis bag and therefore rather short polypeptides, which are inclined to multimerization. These specific properties we use for purification of NEMP1. A specific feature of NEMP3 and NEMP4 is their activation by carnosine. The data obtained in this study show the efficient ways to protect peptides from degradation in intercellular brain medium and the circumstances affecting the implementation of such protection.

Supported by Russian Basic Investigations Foundation (grants 09-04-01571-a and 12-04-00050-a to M. Mosevitsky).

**SW06.S25–25**

**Study of the mechanisms of antiaggregation activity of a-crystallin and chemical chaperones using a test system based on dithiothreitol-induced aggregation of bovine serum albumin**

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To study suppression of dithiothreitol-induced aggregation of bovine serum albumin (BSA) by a-crystallin (a representative of the family of small heat shock proteins) and chemical chaperones (arginine, arginine amide, arginine ethyl ester, proline) at 45°C, dynamic light scattering, analytical ultracentrifugation, size-exclusion chromatography and asymmetric flow field-flow fractionation were used. The kinetics of unfolding of BSA caused by the reduction of disulfide bonds in the protein molecule was followed by monitoring the increase in the tryptophan fluorescence using stopped-flow method. The time of half conversion for the process of BSA unfolding was found to be 26 s. The kinetics of dithiothreitol-induced aggregation of BSA was registered by measuring the increment of the light scattering intensity at 632.8 nm. To calculate the initial rate of aggregation (vagg), the initial parts of the kinetic curves of aggregation were described by the quadratic equation relating time. The anti-aggregation activity of a-crystallin and chemical chaperones was quantitatively characterized by analysis of the dependences of vagg on the chaperone concentration. The comparison of the dependences of vagg on the concentration of intact and cross-linked a-crystallin allowed us to make a conclusion that a non-linear character of the dependence of vagg on the intact a-crystallin concentration was due to the dynamic mobility of the quaternary structure of a-crystallin. To characterize the anti-aggregation activity of the chemical chaperones, we used parameter [L][0.5]. The [L][0.5] value is the chaperone concentration at which two-fold decrease in the initial rate of aggregation takes place. Parameter [L][0.5] can be considered as a measure of the affinity of the chemical chaperone to the protein substrate.
Among the chemical chaperones studied, arginine amide reveals the highest anti-aggregation activity (IC50 ~58 mM).

This study was funded by the Russian Foundation for Basic Research (grants 11-94-00932-a, 11-04-01271-a and 12-04-00545-a) and the Program ‘Molecular and Cell Biology’ of the Presidium of Russian Academy of Sciences).

**SW06.S25–26**

Complementary tools supporting comprehensive mapping of protein complexes via affinity capture/mass spectrometry

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Most strategies to explore protein-protein interactions by affinity capture still follow a trial-and-error approach to identify the best conditions for interaction partner co-purification. Moreover, in the case of some model systems (e.g. mammalian cell culture, animal tissue, etc.) the cost of obtaining enough material for extensive testing via traditional approaches can be prohibitive expensive. Our lab has developed and successfully applied a high-throughput protein affinity capture/co-purification screen applicable across multiple model systems, including human cell lines. Our procedure requires a minimal amount of cell material per purification (~10–50× less than typically used in a bench-scale experiment), producing quantities of purified protein complexes that can be visualized by Coomassie blue stain even in the case of proteins of modest abundance (~1000 copies/cell). By testing up to 96 extraction conditions at a time, the chances of finding multiple instances of distinct protein complexes containing the tagged protein of interest are significantly increased, and the time to obtain results is significantly reduced. This tool has proven useful for rapidly revealing protein-protein interaction networks in a reliable and comprehensive way.

Some protein interactions or particular sub-complex configurations may nevertheless elude capture even with extensive experimental tuning. Therefore we have developed a protocol that describes the treatment of cryo-milled yeast and human cell powder with low concentrations of formaldehyde for short time courses, followed by affinity capture of the proteins of interest. The formaldehyde treatment stabilizes interacting proteins with suitable interaction interfaces. This is presumed to occur via the induction of reversible covalent linkages in the form of diverse methylene bridges between directly interacting proteins. As a result, this treatment affords the possibility to detect protein interactions previously unobserved by other means. The reversible nature of the formaldehyde-stabilized material provides for interactions mapping via standard gel-based or in-solution mass spectrometric approaches.

We propose these tools as complementary methods for improving our ability to detect and more completely describe physiologically relevant networks of protein interactions.

**SW06.S25–28**

Interaction of actin-binding protein actinin-4 and actin in the cell nucleus

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During recent years, a number of cytoskeletal proteins abundant in cytoplasm were also found in the nucleus. For example, these include actin, profilin, tubulin, gelsolin, alpha-actinin 4. However their nuclear functions and intra nuclear localization are under extensive investigation. The major component of the cytoskeleton – actin is also an important component of complexes involved in chromatin remodeling, histone modifications, PIC (pre-initiation complex) formation, transcription elongation, RNA processing and gene expression. Furthermore, actin is able to undergo polymerization process inside the nucleus. That is required for actin participation in transcription elongation. We proposed that nuclear actin can act together with its cytoplasmic partner alpha-actinin 4 (ACTN4). ACTN4 in the cytoplasm bundles actin filaments and participates in stress fibers formation. In the nucleus, ACTN4 regulates gene expression and may potentially be involved in splicing control via cooperation with hnRNP complexes. Despite nuclear localization of both actin and ACTN4, there are no data about their interactions within the nucleus. Analysis of nuclear actin-ACTN4 interactions is a challenging task due to high abundance of both proteins in the cytoplasm and tight attachment of actin structures to the outer side of the nuclear envelope. Using the confocal microscopy combined
with nuclear isolation technique, and digitonin treatment of the cells, we intend to resolve this problem. Since the action participates in formation and function of the BAF chromatin remodeling complexes, we hypothesize that interactions between actin, ACTN4 and these complexes may also occur. To test the proposition we selected a BAF155 actin-associated subunit of BAF complex.

This work was supported by grant of Russian Foundation of Basic Research (12-04-32194 MOL_A, 13-04-00497 A_2013), Russian Government Programme for the Recruitment of the leading scientists into the Russian Institutions of Higher Education (11.G34.31.0069).

SW06.S25–29
Comprehensive analysis of proteome changes induced by the inactivation the tumor suppressor ACVR2 in microsatellite unstable colon carcinoma cells

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The activin type II receptor (ACVR2) gene is a tumor suppressor gene that is frequently mutated in microsatellite-unstable colon cancers (MSI-H colon cancers). ACVR2 is a member of the transforming growth factor (TGF)-type II receptor (TGFBR2) family and controls cell growth and differentiation. We applied an ACVR2-deficient MSI colon cancer cell line (HCT116) to set up an experimental design for comprehensive analysis of proteomic changes associated with such functional loss of a tumor suppressor. We established a stable and doxycycline inducible cell line expressing functional TGFBR2. The generation of this cell line involved a strategy called recombinase-mediated cassette exchange (RMCE). The obtained cell line has been characterized comprehensively. Both, ACVR2 expression and subsequent Smad signaling were analyzed by RT-PCR and Western blotting. These results imply that ACVR2 protein is present and functional. To study ACVR2 dependent effects on protein expression, we performed metabolic labeling experiments with l-azidohomoalanine using our modified cell line. The resulting metabolically labeled azide-modified proteins are reacted with carboxamide-6-azido-280rene petri dishes (60 × 15 mm) coated with purified N terminal 6xHis-TerB proteins. Three cycles produced a pool of specific peptide sequences that bound to the TerB protein. From each cycle, a number of phage clones were purified and sequenced for characterization. The peptide sequences of the final elutions were then compared for possible sequence homologies through BLAST (http://www.ncbi.nlm.nih.gov).

We found several peptides representing apparent consensus sequences, AHSSANFDVKGI (15/103 or 15.45%), AL-WPPNLHAWVP (9/103 or 9.27%), YDAHNYGDGSP (3/103 or 3.09%), TMGYTQRLVYT (2/103 or 2.06%), TPMVERNYNAAD (2/103 or 2.06%) and MPLMSEPALEML (2/103 or 2.06%) that may be part of the interactions partners, or their domains for expressed TerB protein. Based on these results, several proteins can be tested in vivo by two-hybrid system.

Acknowledgements: This study was realized with financial support of the project: ‘The Development of the Center of Excellence for Exploitation of Informational Biomacromolecules for Improvement of Quality of Life (ITMS: 26240120027) and also by the Grant UK/118/2012.

SW06.S25–30
Exploring protein-protein interactions of the TerB protein with phage display

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The objective of this study was to identify protein-protein interaction partners for TerB protein, one of the essential tellurite resistance proteins, using phage display techniques. The 12-mer random peptide phage library provided from New England Biolabs was used for selection of TerB protein-specific peptides. Three rounds of panning were done in individual sterile polystyrene petri dishes (60 × 15 mm) coated with purified N terminal 6xHis-TerB proteins. Three cycles produced a pool of specific peptide sequences that bound to the TerB protein. From each cycle, a number of phage clones were purified and sequenced for characterization. The peptide sequences of the final elutions were then compared for possible sequence homologies through BLAST (http://www.ncbi.nlm.nih.gov).

We found several peptides representing apparent consensus sequences, AHSSANFDVKGI (15/103 or 15.45%), AL-WPPNLHAWVP (9/103 or 9.27%), YDAHNYGDGSP (3/103 or 3.09%), TMGYTQRLVYT (2/103 or 2.06%), TPMVERNYNAAD (2/103 or 2.06%) and MPLMSEPALEML (2/103 or 2.06%) that may be part of the interactions partners, or their domains for expressed TerB protein. Based on these results, several proteins can be tested in vivo by two-hybrid system.

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Macromolecular crowding is expected to favour protein-protein interactions in the cell, in particular aggregation processes. Chaperones are able to prevent aggregation, however the mechanisms of their action under crowding conditions are poorly investigated.

We studied the functioning of α-crystallin, a member of sHsps family, and chemical chaperone, prolinc, under crowding conditions at 37°C using test systems based on aggregation of UV-irradiated glycogen phosphorylase b (Phb) (system I), dithiothreitol-induced aggregation of α-lactalbumin (system II) and aggregation of apophb (system III). The study of the aggregation kinetics showed that the antiaggregation activity of α-crystallin decreased under crowding conditions. Such conclusions were drawn on the basis of the light scattering intensity studies. However analytical ultracentrifugation and other methods showed that the interaction of chaperones with protein-targets was retained under crowding conditions. The data obtained in systems I and III are indicative of the existence of two types of α-crystallin-protein-target complexes differing in the sensitivity to crowding. High order complexes reveal high propensity to aggregation under crowding conditions, while the complexes comparable to α-crystallin in size and smaller complexes are resistant to aggregation in the presence of the crowding agents. Crowding stimulates the rate of aggregation of the high order complexes resulting in the decrease in the above-mentioned chaperone-like activity of α-crystallin.

We assume that the chaperone-like activity of α-crystallin under crowding conditions is connected with the formation of the complexes between the protein-target and the dissociated forms of α-crystallin.

Using reconstruction of holophb as an example, we studied the participation of chaperones in the processes of reconstruction of hisozenzymes from apoform and cofactor under crowding conditions. It has been shown that chaperones favour the formation of small oligomeric forms of apophb resulting in the acceleration of the reconstruction process.

This study was funded by the Russian Foundation for Basic Research (grants 11-04-01271-a and 11-94-00932-a) and the Program ‘Molecular and Cell Biology’ of the President of Russian Academy of Sciences.
SW06.S25–32
Modifications of the acidic soluble salivary proteome in human babies from the birth up to the age of 48 months investigated by a top-down HPLC-ESI-MS platform

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The salivary proteome comprises proteins secreted by the major and minor salivary glands, as well as proteins originated from gingival crevicular fluid, plasma exudation, desquamated epithelial cells and host oral flora [1]. The major components of gland origin and distinctive of human saliva are: histatins (Hst), stathemin, P-B peptide, S-type cystatins, acidic and basic proline-rich proteins, amylases, and mucins [2]. Age-related differences in the human salivary proteome have been recently demonstrated in pre-term newborns [3] and in subjects aged between 3 and 44 years [4]. The present study extends these previous findings and investigates the age-related differences in the acidic soluble salivary proteome in human babies from the birth up to the age of 48 months. Eighty-nine healthy babies were enrolled for the study and divided according to the age into five groups. Among the proteins analyzed, the levels of acidic proline-rich proteins, S-type cystatins, and P-B peptide increased with age. A particular age-related trend was observed for Hst 1, that reached a maximum of concentration in the 7–12 months old babies followed by a drastic decrease in the 13–24 months old babies. The overall age-related variation of Hst 1 may be connected to the primary dentition eruption.

References

SW06.S25–33
Monoclonal antibodies to alfaC-regions of fibrin(ogen)

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The central protein of the blood coagulation system is a fibrinogen, which consists of central region E, two peripheral regions D and two αC-regions (Aα220-610). Fibrinogen is transformed into fibrin by thrombin. At present, special orientation of αC-regions in fibrin molecule and its role in fibrin network formation are unknown.

Monoclonal antibodies (mAbs) FnII-2M, I-5B and I-6B to αC-region of human fibrinogen(ogen) have been obtained. The epitope for mAbs FnII-2M, I-5B are localized in the fibrin fragment Aα240-491 and for mAb I-6B – in Aα509-602 fragment. By virtue of mAb FnII-2M, which react only with fibrin desAβ it was found that αC-regions are associated with fibrinopeptides B (FpB) in the fibrinogen, monomer and polymer fibrin desAβ. However, the αC-regions move away from the core of the molecule after FpB cleavage by thrombin from fibrin desA proteolysis, leading to enhancing lateral association of protofibrils. Three-dimensional structural computer models reflecting of αC-regions spatial orientation in fibrinogen, fibrin desA and fibrin desAB were constructed on the basis of our results and the literature data.

It was investigated that mAbs I-5B and I-6B decreased the maximum rate of fibrin desAB polymerization up to 90% and 76.2%, respectively at their equimolar ratio to fibrin. These results suggest that these mAbs-inhibitors block the sites of αC-region, which take part in protein-protein interactions during fibrin polymerization.

The obtained mAbs I-5B and I-6B may be used as a «tag»-antibodies together with fibrin-specific «catch» mAbs I-3C1 [1] for quantification of the earliest forms of soluble fibrin in human blood plasma with the aim of early diagnostics of the threat of thrombus formation.

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SW06.S25–34
Peptide mediated targeting of natively unfolded protein synuclein-gamma to inhibit human endometriotic lesions in a xenograft mouse model

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Endometriosis is a painful female reproductive disease in which tissue from the uterine lining (endometrium) migrates outside the womb and implants in other areas of the body. The disorder, for which there is no absolute cure, affects over 200 million women and girls globally. Endometrium in ectopic sites must establish its own blood supply to supports its growth and development into an endometriosis lesion. Natively unfolded protein, Synuclein-gamma (SNCG) has elevated expression in endothelial cells of endometriosis lesions compared to eutopic endometrium. SNCG induces cellular proliferation by inhibiting the mitotic checkpoint kinase BubR1, and modulates estrogen receptor signalling by driving the transcription of estrogen receptor-alpha. Our specific objectives were to assess the effect of a SNCG peptide inhibitor on endometrial epithelial and endothelial cell proliferation in vitro and human endometriotic lesion development in vivo using a xenograft mouse model. The SNCG peptide inhibitor, termed ST011, is composed of a 10 amino acid cell penetrating TAT sequence, and a 12 amino acid SNCG binding sequence. Co-immunoprecipitation demonstrated that ST011 specifically binds to SNCG. ST011-FITC penetrated endometrial epithelial carcinoma cells (EEC) as it was specifically localized within these cell types in vitro after an 8 h exposure. ST011 reduced human umbilical vein endothelial cell (HUVEC) and EEC proliferation in vitro. Human endometriotic lesions, surgically engrafted into the peritoneal cavity of female alymphoid Rag2(−/−)Il2rg(−/−) mice, had reduced blood vessel growth when given daily intraperitoneal injections of ST011 compared to phosphate buffered saline control. Preliminary CD31 immunofluorescence of human endometriotic lesions revealed that microvessel density was lower in ST011-treated lesions compared to phosphate buffered saline control. In a separate experiment, ST011-FITC injected into the peritoneal cavity of alymphoid female mice surgically induced with human endometriotic lesions was uptaken by cell types in endometriotic lesions when visualized using intravital confocal microscopy. These in vitro and in vivo studies indicate that the targeted inhibition of
Proteomic profile of placenta during physiological pregnancy and preeclampsia

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Background: Development of pregnancy depends on metabolic usefulness of the placenta, which determines normal functioning of the fetoplacental system during the whole period of the intrauterine ontogenesis. Proteomic analysis of placental tissue will contribute to the elucidation of the molecular mechanisms of pregnancy and related complications, such as preeclampsia, which still is one of the leading causes of maternal and perinatal morbidity. The aim of this study is the analysis of proteome differences between normal and preeclamptic full-term placentas.

Methods: Full-term placentas were obtained after delivery (38–40 weeks of gestation) from women with physiological pregnancy (n = 10) and with preeclampsia (n = 10). The expression of placental protein was analyzed using proteomic approach. Proteins were fractionated by two-dimensional gel electrophoresis (1st dimension: IPG-strip, pH 3–10, 17 cm; 2nd dimension: 8–16% polyacrylamide gel). Some proteins of interest were excised from the gel, digested and identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Results: Statistical analysis indicated that 30 proteins were differentially expressed in preeclamptic compared with normal placentas (p < 0.05). Eighteen proteins were successfully identified by MALDI-TOF-MS, including molecular chaperone, signal transduction protein, cytoskeleton and membrane proteins, proteins involved in the oxidative metabolism. Among them, seven proteins were up-regulated (endoplasmnin, aconitrate hydratase mitochondrial, 14-3-3 protein epsilon, peroxiredoxin-4, alpha-actinin-4, tropomyosin beta chain, hsp60 mitochondrial) while six decreased expression (annexin A4, actin cytoplasmatic, tropomyosin alpha-1 chain, actin-related protein 2, 3 complex subunit 2, proteasome subunit alpha type-6, 60S acidic ribosomal protein) in preeclampsia in comparison with normal placentas.

Conclusion: Results of the present study suggest that development of preeclampsia is characterized by altered production of protein playing important regulatory functions. These differences in placental proteome, obviously, have pathogenetic importance for the development of preeclampsia and can be used for predicting the course of the neonatal period.

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The role of meizothrombin and prethrombin-1 in fibrin formation and platelet aggregation

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Meizothrombin (MT) and prethrombin 1 (P-1) are prothrombin derivatives, which appear in the haemostasis activation area. Gladomain and a catalytic domain with fully formed active centre so MT possesses thrombin-like functions. P-1 consists of a kringle domain and an inactive catalytic domain so P-1 has no enzymatic activity and can not bind to membrane, but it has formed exosite I.

However, MT and P-1 role in haemostasis regulation isn’t clear. So we studied the role of MT and P-1 in fibrin formation, platelet activation and aggregation.

A new effective method of obtaining of MT from native human prothrombin was developed. Immobilized prothrombin activator from Echis multisquamatis venom was used for its obtaining. The preparation was stable and electrophoretically pure.

MT was able to transform fibrinogen to fibrin and activate clotting factor XIII, but less effective than thrombin. It didn’t activate washed platelets; but activated heterogeneous suspension of washed platelets with a pool of activated cells. MT was able...
Various software packages, including MapThis and tunica media of the aorta, as well as two additional modules containing data on human proteins from myocardium, skeletal muscle, and many organs and the most significant in the brain.

P-1 did not promote fibrin formation or activate platelets. However, it dramatically affected adrenalin-induced platelet aggregation and prolonged lag-phase of collagen-induced platelet aggregation in platelet rich plasma. It also strongly reduced speed of ADP-induced aggregation of platelets in platelet rich plasma, decreased aggregation rate and promoted the appearance of the 1-st aggregation wave. Thus we can assume that P-1 is formed to prevent overproduction of thrombin and hold up clotting cascade because it has no enzyme activity and does not turn into thrombin. Since it also decreases platelet aggregation, it probably has a strong effect on clotting regulation.

Thus, both studied prothrombin derivatives were shown to be able to act on crucial coagulation processes – fibrin formation and platelets aggregation. We can conclude the regulatory role of MT and P-1 in pathological processes during inflammation, atherosclerosis and intravascular clotting.

**SW06.S25–38**

‘Muscle organs proteomics’ multi-level database

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As a result of long-term proteomic studies [1,2,3] and with the aim to optimize the further investigations the ‘Muscle Organs Proteomics’ database was created (MOP, http://mp.inbi.ras.ru). The earlier versions consisted of five interrelated modules, containing data on human proteins from myocardium, skeletal muscle, myometrium, prostate (that contains smooth muscle cells) and tunica media of the aorta, as well as two additional modules (proteins of myoblasts and rhabdomyosarcoma cultured cells). Various software packages, including MapThis! Molly Penguin Software, Melanie ImageMaster, Mozilla Firefox and some others were used for MOP forming.

MOP contained data about 510 protein fractions that were obtained using proteomic technologies (O’Farrell 2DE in NEPHGE, IEF and IPG variants; digital image analysis; protein identification with MALDI-TOF MS and Western blot).

The analysis of collected material allowed to identify 22 electrophoretic isofoms of transgelin proteins in tissues containing smooth muscle cells. At the same time, the patterns of transgelin isoforms were quite specific for different organs, that allows to consider them as potential tissue-specific biomarkers.

New version of MOP (2013) includes two more information modules, which contain the results of proteomic studies of muscles of two animal species that are used in livestock and meat industry (‘Proteins of skeletal muscle of Sus scrofa’, ‘Proteins of skeletal muscle of Bos Taurus’). During the comparative analysis a number of species-specific protein isoforms (β-enolases, triose-phosphate isomerases, troponins, myosin light chains) was revealed. Thus, the new version of the MOP database opens up the opportunities for task-oriented study of species specificity of muscle proteins with the prospects of control the quality of raw meat and meat products.

**References**


**SW06.S25–39**

Rat hippocampal proteomic alterations following acute emotional stress

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Stress is a significant factor that we and all living organisms encounter throughout life. Depending on the type of stress, there are number of mechanisms to keep the body homeostasis or to minimize stress effects. Stress causes physiological changes in many organs and the most significant in the brain.

To identify the biochemical changes induced by acute emotional stress a proteomic level, we compared the hippocampal proteome of rats before and after acute emotional stress. Experiments were performed on male Wistar rats aging 10 weeks. The animals were randomized into two groups (40 specimens each) with different prognostic emotional resistance (active and passive rats). Each of this group was divided into four subgroups: control, stress, 1st day and 3rd day after stress. Twelve-hour immobilization of animals was used as a model of stress. Rat hippocampus from the brain of each animal in all eight groups was removed and analyzed using 2DE technique. Using PDQuest advanced 2-D analysis software, approximately 112 proteins were detected in the 2D gels, among them 15 proteins exhibited differential expression. Proteins of interest were identified by MALDI-MS peptide mass fingerprinting using Mascot search against the NCBI nr database.

In the hippocampus, 15 spots showed significant variation between treatment groups. Phosphatidylethanolamine-binding protein, thiomorpholine-carboxylate dehydrogenase and glutamine synthetase were not present in passive rats during the stress; prohibitin wasn’t present in passive rats in control group. Acute stress reduces the expression of alpha-synuclein in hippocampus of both passive and active rats and induce the expression of F-actin-capping protein. Three proteins had restricted level in hippocampus of active animals during the all experiment. Among them was Cu-Zn superoxide dismutase. Calreticulin was reduced in recovery groups (1st and 3rd days after stress) as compared with controls and stress. Our results demonstrate that isoamyl acetate-hydrolyzing esterase is highly expressed in active rats of control group and during the stress, and in passive rats during the recovery period (3 days). These results indicate that the proteome patterns from the hippocampus of stressed rats differ significantly from the control (unstressed) group.

Our findings indicate an activation of a number of cellular mechanisms that play important roles in the maintenance of adaptation potential of organism. Proteomic features of emotigenic structures of the brain (i.e. hippocampus) in animals with different emotional reactivity probably contribute to the existence of significant variations in the individual resistance to emotional stress.
**SW06.S25–40**

**On the role of αC-regions of fibrin in the self-assembly and lateral association of protofibrils**

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αC-region is formed by the C-terminal portion of αA-chain of fibrinogen (Fg) containing 220–610 amino acid residues. After FpAs cleavage and formation of fibrin desA oligomers connection between FpA and αC-domain and interdomain αC-domain–αC-domain connection are broken down. In this case, αC-domains remain bound to BjN-domain of E-region of fibrin desA molecules. As a result BjN-domain and αC-domain form a new temporary structure. In this study, we investigated the role of the BjN-domain–αC-region complex in the self-assembly and lateral association of fibrin protofibrils.

Fg was isolated from the donor plasma, X-, αC-, D- and DD-fragments were isolated from Fg. Electrophoretic and ELISA assay with mAb to αC- and BjN-domains, respectively, showed that X-fragment did not contain a fragment of αC-regions, but retained all BjN-domains. D- and DD-fragments were purified with fibrin-sepharose. It was found that the X-fragment polymerization, activated by thrombin, was much slower of the Fg one: slowing the rate of protofibrils formation 5.2 times, the speed of lateral association – by 3.2 times, and the resulting turbidity of the clot – by 2.4 times. The rate of fibrinopeptide A cleavage by thrombin from X-fragment was also slightly slower. Twenty-four kDa fragment of αC-region inhibited fibrin polymerization, but did not affect the polymerization of X-fragment. Comparison by turbidimetric method the Fg and X-fragment polymerization, initiated by thrombin and reptilase, showed that in the system Fg+Thr, X+Thr and X+reptilase D- and DD-fragments inhibited fibrin and X-fragment polymerization. However, in the system Fg+reptilase DD-fragments had weak inhibitory effect and D increased the rate of fibrin polymerization.

Taking into account that only fibrin desA, which is formed in the system Fg+reptilase has complex BjN-domain–αC-region, we can assume that this complex contributes into the self-assembly of fibrin desA molecules in protofibrils, stabilizes protofibrils and promotes the protofibrils lateral association.

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**SW06.S25–41**

**Assessment of blood contamination in biological fluids using MALDI-TOF MS**

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**Introduction:** Screening biological samples for blood contamination is crucial for proteomics analysis because blood proteins may bias the final results. Visual inspection, counting red blood cells, and measuring blood derived proteins (apolipoprotein B, hemoglobin (Hb)) are the most often used techniques for detecting blood contamination in biological fluids. Cerebrospinal fluid (CSF) and follicular fluid (FF) are two body fluids, extensively used in proteomics, which may become contaminated during collection procedure. An effective and sensitive method is needed for screening biological fluids against contamination before further proteomics analysis.

**Results:** A rapid and sensitive MALDI-TOF MS method based on Hb detection for the assessment of blood contamination in biological fluid samples was optimized. Screening of CSF samples revealed distinct Hb peaks from 3 of the 25 inspected CSF samples, indicating the blood contamination in these samples. The different intensities of the Hb peaks in these MS spectra may indicate that these CSF samples were contaminated by blood at slightly different levels. The detection limit of MALDI-TOF MS based blood contamination detection assay was as low as 0.12 nM (S/N ratio of 6.8), which was found to be 5.8-fold more sensitive compared to UV spectroscopy. In addition, optimized MALDI-TOF MS based method needs significantly smaller sample volume compared to UV-method (1 μl compared with 50 μl, respectively), which is a considerable benefit if one is to analyze ‘hard-to-obtain’ biological fluid samples. To confirm the results obtained on CSF samples, we applied the MALDI-TOF MS based contamination control assay to screen 130 FF samples prior proteomic analysis. Hb α- and β-chain peaks were detected in 31 of the 130 screened FF samples.

**Conclusions:** We optimized a rapid, robust, and low sample volume needing MALDI-TOF MS method based on Hb detection for blood contamination exclusion, which could be routinely used before proteomics analysis of any biological fluid. We demonstrate that the technique can be successfully applied to trace blood contamination in cerebrospinal fluid in neurology and follicular fluid in reproductive medicine.

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**SW06.S25–42**

**Purification of the YgdP Nudix protein, a putative virulence factor from Pseudomonas aeruginosa**

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**Pseudomonas aeruginosa** is an opportunistic human pathogen that frequently causes hospital infections. The observed difficulty in treating infection caused by this microorganism is due to its high intrinsic and acquired resistance against a wide range of antibacterial agents. Thus, there is a need for search of novel factors involved in *P. aeruginosa* pathogenesis e.g. Nudix proteins and consequently for novel approaches for controlling these infections. Nudix proteins, widely distributed among all classes of organisms, belong to a class of pyrophosphatases. These enzymes catalyze the hydrolysis of a variety of nucleoside diphosphate derivatives including (d)NTPs, NTPs (canonical and modified), nucleoside sugars (e.g., ADP-ribose), various coenzymes, alarmone type molecules ApA, and m3GTP mRNA cap.

This work focuses on purification of the YgdP Nudix protein from *P. aeruginosa*. Orthologs of this Nudix hydrolase were identified in other pathogenic bacteria such as *E. coli K1*, *Legionella pneumophila* and *Pasteurella multocida*, and shown to be associated with their invasiveness. In order to analyze the YgdP biochemical properties attempts to purify this hydrolase were undertaken.

The *PA0436* gene coding for the YgdP protein was expressed as a fusion with His-tag encoding sequence. Regardless of the expression settings used, the majority of the recombinant His-YgdP protein was insoluble in either *E. coli* or in *Pseudomonas aeruginosa*. Such accumulation of protein in insoluble fraction might indicate a toxicity of the expressed protein. Moreover, we observed the presence of the chaperone protein Hsp90 in the slight amount of the recombinant protein, that remained in soluble fraction, suggesting folding problems of the expressed YgdP protein.

The observed difficulties with YgdP purification while using the methods commonly used forced us to search for alternative purification methods. One of the chosen approaches employs the anionic detergent N-lauroyl sarcosinate (Sarkosyl).
Our preliminary results indicate that this new method will allow us to obtain the YgdP protein in a quantity and quality required for its further biochemical analysis.

**SW06.S25-43**

**Production of human recombinant His-tagged prethrombin-2 in *Escherichia coli* expression system and its activation to thrombin**

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Thrombin, a key enzyme of blood coagulation, currently received increasing interest in biopharmaceutical and biomedical research and it is widely used in biotechnology and pharmaceutical industry as a topical hemostatic agent in surgery. Optimal way to prepare pathogen-free human α-thrombin, with respect on strict requirements to be used as a pharmaceutical reagent applied in clinical practice is based on techniques of recombinant DNA. Recombinant human thrombin has the advantage of being minimally antigenic and devoid of the risk of viral transmission in clinical applications. *Escherichia coli* expression system remains to be one of the most preferred bacterial expression systems and often is the first choice for the production of industrial and therapeutic recombinant proteins. It offers many advantages, including its inexpensive carbon source requirements for growth, rapid biomass accumulation, ability to high-cell density accumulation and simple process scale up.

In this work we have designed and prepared a proper *Escherichia coli* expression system for the production of human recombinant thrombin. We have optimized the heterologous expression of His-tagged prethrombin-2, the smallest single-chain immediate precursor of α-thrombin, in Erlenmeyer flask and batch cultivation in fermenter. The expressed prethrombin-2 was found to be insoluble and formed intracellular inclusion bodies from which the protein was partly refolded and next purified by using immobilized metal ion affinity chromatography (IMAC). In the next step we would like to activate those modified prethrombin-2 to active thrombin using a snake venom ecarin.

This contribution is the result of the projects implementation: ‘Industrial research of new drugs based on recombinant protein’ ITMS 26240220034 and ‘Development of Centre of competence for research and development in molecular medicine’ ITMS 26240220071 supported by the Research & Development operation Programme funded by ERDF.

**SW06.S25-44**

**Production of recombinant human enterokinase light chain in methylotrophic yeast *Pichia pastoris***

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Enterokinase, a type II trans-membrane serine protease, highly efficiently catalyzes the hydrolysis of the polypeptide chain downstream of the N-terminal sequence (Asp)4Lys. Its high specificity for the recognition site and tolerance to a wide range of reaction conditions are the main characteristic features that make enterokinase an useful tool for an *in vitro* cleavage of various fusion proteins containing the sequence (Asp)4Lys (the enterokinase linker) inserted between the carrier and the target protein. Enterokinase is synthetized as a single-chain (zymogen), whereas the mature active form of enzyme is comprised of light chain and disulfide linked heavy chain. The heavy chain anchors the enzyme in the intestinal brush border membrane and the light chain represents catalytic domain of serine protease.

In the beginning, our aim was to develop a highly effective expression system for production of recombinant human enterokinase light chain (rhEKL). We chose eukaryotic host strain of methylotrophic yeast *Pichia pastoris* suitable for a shake flask and a laboratory scale fermenter. *P. pastoris* as an eukaryotic organism provides post-translation modifications (e.g. disulfide bond, glycosylation) required for functionally, correctly folded recombinant protein. Proteins are usually secreted into growth medium in soluble form, which makes purification of secreted proteins easier, because of relatively low levels of endogenous proteins in the extracellular medium. We achieved higher expression level of rhEKL in comparison to the host organism *E. coli*. Next step was the purification of rhEKL by taking advantage of the His-tag at the C-terminal part of the coding sequence. Recently, this study has been focused on testing enzymatic activity of rhEKL on synthetic peptide Gly-Asp-Asp-Asp-Asp-Lys-Lys-naphthydamide.

This work is the result of the project implementation: ‘Production of biologically active agents based on recombinant proteins’ (ITMS 26240220048) supported by the Research and Development Operational Program funded by the ERDF.

**SW06.S25-45**

**Study of *B. burgdorferi* outer membrane proteins by protein-protein interaction approach**

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*Borrelia burgdorferi*, the causative agent of Lyme disease is transmitted from infected *Ixodes* ticks to a mammalian host after a tick bite. During the transmission process spirochetes must adapt to very different conditions which is achieved by pronounced change of the expression of several outer surface proteins. In addition, many of these proteins are thought to be important for the establishment and maintenance of the infection by exploitation of various pathogenic mechanisms.

In this study, we applied a targeted protein-protein interaction based pullout proteomic strategy as an initial screen to identify possible *B. burgdorferi* outer surface protein interaction partners in the mammalian tissues. For this purpose *B. burgdorferi* proteins were expressed in *E. coli* cells as 6XHIs tagged recombinant proteins, coupled to the nickel agarose magnetic beads and probed with a set of tissue lysates. After SDS-PAGE analysis protein bands were identified by mass spectrometry. Spectrometry data sufficient for the further analysis were obtained for 12 visible protein bands. The analysis of the peptide profile revealed ten possible interaction partners for two borrelia proteins studied, thus these data could help to elucidate the unknown biological function of *B. burgdorferi* proteins.

Our results demonstrate the general utility of this methodology in the protein-protein interaction studies of molecular mechanisms of Lyme disease.
Antimicrobial peptides (defensins, cathelicidins etc) are crucial molecules of host defense in animals. Our experimental data contributed to the concept that the defensin superfamily (alpha-, beta-, theta-defensins and insect defensins) is the most ubiquitous group of antimicrobial peptides. We have discovered the members of this group of peptides in jellyfish Aurita aurita (Type Cnidaria) named aurelin. Aurelin has structural features of defensins and neurotoxin peptides. Our investigations resulted in the discovery of the novel beta-defensin peptides in the turtle (Emys orbicularis) and chicken (Gallus gallus) leukocytes. In the course of the research of antimicrobial molecules in monkeys Macaca mulatta and Papio hamadryas several families of the members of alpha- and theta-defensins have been revealed in blood leukocytes: These data provide evidences that the defensin superfamily is the most prominent group of the defense peptides in animal evolution. However, some animal species have a lack of defensins in their phagocytes. For example, we have shown that the coelomocytes of the sandworm Arenicola marina contain arenicins which include one disulfide bond in their molecules and adopt a beta-hairpin conformation. The members of the defensin superfamily are also absent in porcine, ovine, caprine, canine blood leukocytes. We have isolated and sequenced an array of the antimicrobial peptides of the cathelicidins family (pig protegrins and prophenins, goat and sheep bactenecins). The specific set of antimicrobial peptides has been found in the leukocytes of sturgeons, these peptides named acipensins are histone derived from the terminal fragments of H2A histone. The peptides isolated from the leukocytes of the stellate sturgeon were structurally identical to Ac2 and Ac3. We investigated the biological activity of three major peptides of the Russian sturgeon (Ac 1, 2, 6). Ac1 and Ac2 exerted potent antimicrobial activity towards Gram-positive and Gram-negative bacteria in the radial diffusion assay, whereas Ac6 was effective only against E. coli. All three sturgeon peptides did not cause appreciable hemolytic effect on human erythrocytes up to 40 mM concentration of the peptides and their toxicity toward other eukaryotic mammalian cells was also insignificant. By the date several AMPs derived from histone H2A have been described in literature including hipposin from the mucus of Atlantic halibut (Birkemo et al., 2003) and buforin (Kim et al., 1996) from amphibian skin. Our findings support the idea that histone-derived peptides may serve as important host defense molecules of blood leukocytes in sturgeons.

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C91/PL after heparin treatment. As analyzed by comparative PAGE, the pattern of biofilm proteins included some major bands, which were not observed in samples of purified viral particles. These protein bands were excised and analyzed by MS. We found in biofilm preps some proteins of cytoskeleton – actin, annexin A6 and vimentin. Surprisingly, these proteins were expressed on the surface of HTLV-1 infected cell lines MT2 and C91/PL, but not on the surface of uninfected T cells. We also generated mAbs against HTLV-1 biofilms and selected those, which colocalized with the viral assemblies on the surface of C91/PL cells. In summary, we have partially identified cellular proteins that compose viral biofilms by using MS-analysis, and generated a panel of mAbs against biofilms. The further identification of generated mAbs will expand our knowledge of biofilm composition, while gene expression or knockdown experiments will shed light on the role of biofilm components in cell-to-cell transmission of HTLV-1.

**SW06.S25–49**

**Profiling of human neuroblastoma SH-SY5Y cells cytokines/chemokines secretome by Luminox xMAP multiplex assay**

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The human neuroblastoma SH-SY5Y cell line has been widely used in experimental neurobiological studies, including neuronal differentiation, metabolism, and function related to neurodegenerative processes, neurotoxicity and neuroprotection. SH-SY5Y cells can differentiate in a functionally mature neuronal phenotype. We use differentiated cells induced by retinoic acid as in vitro model with morphological similarity to living cholinergic neurons in the human brain. To identify cytokines, chemokines and other factors secreted by undifferentiated and differentiated SH-SY5Y cells, we investigated culture condition media from the cells sampled daily during 8 days of cultivation. The undifferentiated SH-SY5Y 0.5–1 × 10^6 cells were seeded on 6-well plates, washed next day, and incubate for 8 days with sampled daily a small aliquots of culture media. The differentiated cells were previously differentiated by retinoic acid during 7 days. The same amounts undifferentiated and differentiated cells were used in the experiment. Twenty-seven cytokines and growth factors were analyzed using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad). Kinetic increase in concentrations during culture growth can be noted for all analytes. Levels of cytokines below 10 pg/ml were demonstrated for number proteins after 8 days of culture in both undifferentiated and RA-differentiated cells (IL1b, IL2, IL4, IL5, IL6, IL9, IL15, eotaxin, G-CSF, GM-CSF, MIP-1α, MIP-1β, RANTES). Other group of cytokines has concentration level below 50 pg/ml after 8 days of culture (IL1ra, IL7, IL8, IL10, IL17, IFN-γ, IP-10, PDGF-bb, TNF-α). Secreted IL12, MCP-1 (MCAF) and VEGF levels were 160–3100, 300–400, 2000–3000 pg/ml in undifferentiated cells and 50–90, 2700–3100, 1400–1500 pg/ml in differentiated cells, respectively. Changes in concentration of these proteins may be important for using in development cell models in neuroimmunology, neurotoxicity and neurodegenerative diseases.

**SW06.S25–50**

**Cis-gamma-amino-l-proline peptides as an example of cell-penetrating peptides**

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The discovery of cell-penetrating peptides (CPPs) is a promising breakthrough to achieve non-invasive delivery of non-permeable biomolecules in the intracellular compartment [1]. CPPs with non-natural amino acids in their sequences (β-amino acids or γ-amino acids) have the main advantage that they show higher stability to proteolytic degradation [2]. Our group designed and synthesized two discrete γ-peptide libraries based on (2S, 4S)-4-amino-l-proline, all compounds having a well-defined secondary structure [3,4]. The libraries were synthesized on solid-phase, using one protected monomer that can be easily functionalized by modification of the a-amino side chain. These peptides can mimic protein interactions and consequently their suitability for diverse therapeutic applications can be explored.

Using flow cytometry and confocal microscopy, here we studied the capacity of these γ-peptides to cross cell membranes in two distinct systems, namely HeLa cells and Leishmania as models of mammalian cells and a parasite, respectively. The subcellular localization experiments were performed under a range of peptide incubation conditions and with a variety of fluorescent labels that tagged cytoplasm, mitochondria, lysosomes, endoplasmic reticulum and Golgi apparatus in HeLa cells. Furthermore, we addressed the uptake mechanisms of these γ-peptides by means of inhibitors of specific endocytotic pathways.

Our results showed that one and two specific peptides are taken up in HeLa cells and Leishmania model, respectively, and that they show some remarkable differences with respect to TAT. The TAT peptide is considered a gold standard in the field. In additionally, the γ-peptides showed low cytotoxicity and protease resistance. We propose that the cellular uptake mechanism of γ-peptides involves two or more pathways in the experimental conditions tested.

**References**


**SW06.S25–51**

**Quantitative LC-MS/MSALL discovery of serum peptide biomarkers**

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New highly specific and sensitive disease biomarkers are one of the best known things that are promised as a result of proteomic researches. The most accepted way for this is the analysis of differ-
ent human biological liquids aimed at discovery of proteins that are either unique or have very different concentration in disease samples in comparison with the healthy control. Meanwhile a great deal of information can also be found in native peptidomes containing all sorts of regulatory peptides as well as products of native protein degradation (degradome) reflecting internal protease activity. However quantitative native peptide studies are much rarer due to enormous peptidome complexity and flexibility while the general applicable for human peptidome quantitative MS technique of MultipleReactionMonitoring has very low productivity.

We analyzed serum peptidomes of the patients with colorectal cancer, ovarian cancer and healthy donors. Our aim was not the comprehensive peptidome analysis but rather the possibility to obtain quite complex native peptide samples suitable and reproducible enough for quantitative analysis. The serum samples were prefraccionated on MB-WCX and heated for 15 min at 95°C, simplifying the composition and releasing the peptides absorbed on the surface of serum proteins. The samples were analysed by RP-LC-MS/MS in identification mode with 25 MS/MS spectra per second using 2 h gradient giving 1800–2500 unique peptides (from 90 to 110 proteins) per run. In total about 3500 unique peptides (global FDR 1%) from 115 proteins were identified. These peptides were used for further data-independent SWATH LC-MS/MS quantitative analysis. As a result we found that just 73 peptides from 30 proteins quantitatively distinguished ovarian cancer and 36 peptides from 18 proteins distinguished colorectal cancer from the corresponding generalized control (healthy donors plus the other disease) with the three times and more difference in concentration, while the number of peptides with reliable XIC quantification (p ≤ 0.01 between replicates) was about 1500. Thus the sample preparation procedure suggested was reproducible enough for quantitative DIA SWATH analysis and corresponding peptide biomarker mining.

**SW06.S25–52**

**Heterologous production of recombinant ecarin in Pichia pastoris expression system**

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Ecarin is a component of the venom from the saw-scaled viper *Echis carinatus* that can convert zymogene form of thrombin to alpha-thrombin, an active serine protease with important role of haemostatic reagent. Heterologous expression is used as a tool for increasing its availability and for ensuring the higher purity in comparison with ecarin, which was obtained by isolation from the snake venom. Recombinant ecarin was identified as a promising candidate for replacement of native ecarin in assays utilizing conversion of prothrombin to thrombin. However, very little work has been presented on the characterization of recombinant ecarin, and recombinant enzyme was to date expressed only in cell lines of higher eukaryotes. Yeast host expression had not yet been published. Successful expression in *Pichia pastoris* would not only be broadening the line of hosts capable of active recombinant ecarin production, but could also offer new possibilities in terms of its availability. Ecarin is a potentially very interesting protease with unique properties and could represent an important alternative to proteases used in molecular biology. Here we describe first steps for preparation of production strain of *Pichia pastoris* for inducible and constitutive expression of recombinant ecarin. Suitable DNA sequence was identified to allow expression of functional ecarin in yeast expression system. The focus was on finding of suitable combination of promoter and strain, to maximize the expression rate of the active recombinant ecarin.

**Keywords:** recombinant ecarin, heterologous expression, *Pichia pastoris*.

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**SW06.S25–53**

**Microcin-B-like compounds produced by *Pseudomonas syringae*: structure and species-specificity of antibacterial action**

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Microcin B produced by some strains of *Escherichia coli* is a founding member of the thiazole/oxazole modified microcin (TOMM) family of ribosomally synthesized post-translationally modified peptides. The thiazole and oxazole heterocycles of TOMMS are derived from the cysteine and serine residues of a precursor peptide. Microcin B inhibits DNA gyrase, an essential bacterial enzyme that introduces negative supercoils into DNA. *E. coli* cells producing microcin B contain plasmids carrying a 7-gene mcb operon. The mcbA gene encodes a precursor peptide, the mcbBCD genes encode subunits of the microcin B synthase, an enzyme responsible for converting cysteine and serine residues of a precursor peptide into thiazole and oxazole heterocycles, mcbEF encode an export pump, and mcbG encodes a pentapeptide repeat protein that binds to DNA gyrase, rendering it resistant to microcin B inhibition.

In this work we characterized a microcin B-like compound encoded by *Pseudomonas syringae*, a plant pathogen responsible for the bacterial blight of soybean. We demonstrate that the *P. syringae* microcin B-like compounds are less potent inhibitors of the growth of *E. coli* than microcin B but, unlike microcin B, these compounds are active against various species of the *Pseudomonas* genus, including human pathogen *P. aeruginosa*. Since both the *E. coli* and *P. syringae* microcins are equally active against *E. coli* or *P. syringae* DNA gyrase in vitro, it follows that *E. coli* microcin B is unable to enter *Pseudomonas* cells.

**SW06.S25–54**

**Identification of prions and amyloids by a novel proteomic approach**

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Amyloids represent protein polymers, which consist of fibrils with cross-beta sheet structure. Some proteins form functional amyloid fibrils in physiological conditions; however, most of amyloids are associated with mammalian neurodegenerative and noncerebral diseases. Currently, there are no universal methods for identification of prion and amyloids. It has been shown that amyloids can be separated from other protein complexes by using
ionic detergents that solubilize all or almost all non-amyloid protein interactions. Taking into account this feature of amyloid polymers, we have developed a new technology for proteomic screening and identification of amyloids. This technology involves several steps. First, the amyloid-rich fraction is purified from experimental (amyloid containing) and control (presumably amyloid free) samples by ultracentrifugation and treatment with detergents (sodium N-lauroylsarcosine and (or) sodium dodecyl sulfate). Next, the proteins from experimental and control samples are labeled with Cy5 (red) and Cy3 (green) dyes, respectively. The samples are combined, and the proteins are separated with two-dimensional difference gel electrophoresis (2D-DIGE). The laser scanning allows us to detect proteins presented in experimental sample only (red signal), and proteins that are in both, control and experimental samples (yellow arises due to overlapping of Cy5 and Cy3 signals). As the last step, the detected proteins are extracted from a gel and identified by MALDI TOF/TOF. We tested this antibody-free technology for identification of endogenous yeast prion [PIN\(^{+}\)] (prion isoform of Rnq1p) and polymers of mouse PrP and human A\(^{\beta}\) peptide expressed in yeast cells. This sensitive approach allowed us to identify all amyloids analyzed including Rnq1p, which expresses in yeast at the low level. Moreover, we have shown that Gas1p, Ape1p and Ape4p form detergent-resistant polymers in yeast cells. These data suggest that these proteins are constitutive yeast amyloids. The approach developed may be used for identification of functional or pathological prion and amyloids in any organism.

**SW06.S25–55** Comparative proteome analysis of toxic metal resistance of bacterial pathogens

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Proteomics analysis employing 2DE is an invaluable tool for analyzing differential protein expression patterns with respect to certain phenotype or conditions. In this study we have focused on the tellurite resistance gene determinant – ter operon from uropathogenic strain *E. coli* KL53. Many bacterial strains use genetically encoded mechanisms allowing their growth at high concentration of toxic metal ions. The phenotype of tellurite resistance is particularly interesting for its frequent occurrence in pathogens, e.g. uropathogenic strain *E. coli* O157:H7. Until now, five genetic determinants of tellurite resistance have been characterized in Gram- bacteria. The plasmid encoded resistance determinant – ter operon – was previously isolated from clinical isolate *E. coli* KL53 within the DNA fragment comprising four genes essential for preserving the resistance – *terB*, *terC*, *terD* and *terE*. However, very little is known about the molecular mechanism these genes mediate the resistance thus far. This mechanism was suggested to be associated with many intracellular processes thus warrant on further more complex analyses. In our work we report the 2DE analysis of proteome in total cell lysate from resistant culture of *E. coli* carrying four *ter* genes and wild type control in the presence and absence of potassium tellurite. Using this approach we aimed at providing an insight into differences in protein expression related to tellurite toxicity and tellurite resistance phenotype of bacterial pathogens.

**SW06.S25–56** Characterisation of initiator of replication in *Acetobacter pasteurianus*

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The DnaA is an initiation protein of replication and belongs to the AAA*+* family. It is encoded by *dnaA* gene, located on bacterial chromosome. It plays key role in initiation of replication process. DnaA protein is composed of four domains, each of them has its own specific function [1]. In initiation process protein interacts with specific binding sites on DNA replication origin and additionally it interacts with other proteins as DnaB helicase, DnaC, IHF or HU protein. Exept of initiation of replication it serves as a transcription factor as well [2] and it was found in cell membranes too. It has been studied intensively in *E. coli* strains. In our work we focus on study of DnaA protein in *Acetobacter pasteurianus* 1360 and 1513 cells. We try to compare DnaA protein from *E. coli* and from *Acetobacter* strains. Based on the nucleotide sequence of the genome *Acetobacter pasteurianus* IFO 3281-01 [3], we designed PCR primers to amplify *dnaA* gene. We cloned *dnaA* gene from *A. pasteurianus* to the expression vector, that ensure fusion with a His-tag sequence and enable us to purify this protein. DNA band-shift assays showed that this protein bound specifically to the ORI sequence on DNA. We are going to measure different activities on purified protein, such as ATPase activity or helicase activity. We cloned *dnaA* in vectors with green fluorescent protein too. We are going to use this fusion DnaAGFP and DnaAHisTag proteins in Pulldown assays and Western blots to show how DnaA protein from *Acetobacter* interacts with other replication proteins (DnaB, HU, IHF). And finally we try to compare nucleotide structure and interactions of DnaA protein from *E. coli* and from *Acetobacter* strains.

**References**


**SW06.S25–57** Histidine acid phytase of *Pantoea vagans*

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Phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is the main storage form of phosphorus in plants and accounts for 20–50% of total organic phosphorus in soil. Due to limitation of digestible phosphorus in plant and in animal nutrition it is still common practice to add inorganic phosphorus as plant fertilizer and as an animal feed supplement. A special class of phosphomonooesterases – phytases – are capable of initiating the stepwise release of phosphate from phytate. Due to last 30 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology, so there is a growing interest for new effective phytase-producers.

Phytate-degrading bacteria were isolated on the selective medium containing phytate as a sole source of phosphorus from different soil samples of the Republic of Tatarstan. Species composition of the isolated strains was identified using 16S
rRNA gene sequencing and MLST-analysis. *Pantoea vagans* 3.2 strain was selected for further research.

A periplasmic phytate-degrading enzyme from *P. vagans* 3.2 was isolated and purified about 3600-fold to homogeneity with a recovery of 14% referred to the phytate-degrading activity in the crude extract. The purified enzyme exhibited single pH-optimum at 4.5 and temperature-optimum at 37°C. The Kₘ for the sodium phytate hydrolysis was found to be 0.28 mmol at pH 4.5 and 37°C.

Amino acid sequence of purified phytase was determined via MALDI-TOF mass-spectrometry analysis. Identification of RHGVRPP and HD conservative active site motifs of the histidine acid phosphatases (HAP) in the structure of *P. vagans* phytase allows to relate purified phytase to HAPs class.

High-pressure liquid chromatography (HPLC) analysis established that one and only final product of phytate hydrolysis by *P. vagans* phytase was myo-inositol pentakisphosphate.

Due to the knowledge of enzyme features and phytate hydrolysis pathway, phytase of *P. vagans* is an interesting candidate for agricultural application, as well as for individual inositolphosphate isomers production.

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### SW06.S25–58

**The glucose-regulated protein 78 (GRP78) binding peptide coupled with 111In-labeled polymeric micelles is a novel tool targeting to gastric tumors for improving early diagnosis**

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Increased expression of membrane-bound glucose-regulated protein 78 (GRP78) is considered to be one of the biomarkers for gastric cancers. Therefore, peptides or molecules with affinity to GRP78 can act as a guiding probe to drive the conjugated imaging agents or therapeutic drugs to target cancers. Based on this rationale, GRP78-guided polymeric micelles were designed and manufactured for nuclear imaging detection of tumors. Thiolated GRP78 binding peptide (GRP78BP) was first labeled with maleimide-terminated poly(ethylene glycol)-poly(e-caprolactone) and then mixed with diethylenetriaminepentaacetic acid (DTPA)-linked poly(ethylene glycol)-poly(e-caprolactone) to form DTPA/GRP78BP-conjugated micelles. The coupling efficiency of micelles with radioisotope indium-111 (111In) was measured and analyzed by instant thin layer chromatography. The coupling efficiency of DTPA-conjugated micelles and DTPA/GRP78BP-conjugated micelles with 111In was 85% and 93%, respectively. For characterization and trace imaging, the radioisotope 111In-targeting tumors were detected and imaged in a xenograft murine model using nano single photon emission computed tomography/computed tomography. The results revealed that the radioactive intensity measured in the animals administered with GRP78BP-guided 111In-labeled micelles was statistically higher than that in animals administered with 111In-labeled micelles, demonstrating that GRP78BP more than doubled the accumulation of micelles to the tumor tissue (P < 0.05). The results indicate that the gastric cancer biomarker GRP78 is a probing target in the application of nuclear imaging for tumor diagnosis. This novel GRP78BP-guided micelle agent may be applied in clinical practice to complement the histological diagnosis.

**Keywords:** biomarker, glucose-regulated protein 78, nuclear imaging, gastric cancer, micelles.

### SW06.S25–59

**Purification of apoptosis-inducing protein using 2D cell blot method**

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Many kinds of columns and much time are required for analysis of protein functions. Therefore, the development of new methods that enable high-throughput analysis of protein functions comprehensively is of the utmost importance.

Hayman [1] et al. developed SDS-PAGE cell blot method for the analysis of cell adhesive proteins. In their study, the proteins separated by SDS-PAGE were transferred to a PVDF membrane, and HeLa cells were cultured on the PVDF membrane. After this, unknown cell binding proteins were found by observing the spots where cells attached. However, this method has not been a prevailing technology because SDS is known as a detergent which denatures many kinds of proteins.

In this study, a protein that induces apoptosis in *Pieris rapae* was analyzed using Two-Dimensional Cell Blot method with low concentration SDS. The proteins in body fluid of *Pieris rapae* were separated two-dimensionally (IEF and PAGE), with low concentration of SDS that did not denature protein and transferred to a PVDF membrane, and HeLa cells were plated over the protein-transferred membrane evenly. After overnight culture, a spot with the alteration of cellular morphology was clearly observed. Dead cells found on the PVDF membrane indicated that there was a *Pieris rapae* protein that induces apoptosis in the hot spot. As the results, a method for the comprehensive analysis of apoptosis-inducing proteins was developed using 2D cell blot method. Moreover, only a small amount of proteins (<1 mg) is required for the analysis of protein functions, the proposed method is expected to be applicable widely.

**Reference**


### SW06.S25–60

**Analysis of protein-DNA interactions in process of plasmid DNA replication**

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Acetic acid bacteria are use as host organisms for the production of important proteins and peptides. Plasmids are extrachromosomal DNA elements with characteristic copy numbers within the host. In many plasmid replications is required plasmid-encoded replication initiator, Rep protein. Rep-type proteins bind to tandem directly-repeated sequences (iterons) to establish the initiation nucleoprotein complex. Iteron sequence length, number and spacing between iteron repeats are typical of every replicon. Rep proteins belonging to the family replication initiators exist mainly as dimers, but only Rep protein monomers can initiate replication by binding to repetitive sequences. Rep proteins interact with the regulatory region of the DNA molecule by the secondary structure consist of intermittent alpha-helices (HTH motif). The Rep proteins of some replications, have an important second function, they recognize inversely-repeated sequences (operators) which overlap the promoter of their own coding genes, acting as self-repressors.

We isolated and characterized plasmid pAG20, from cells Acetobacter acetii CCM 3620, who for its replication encodes a small replication protein Rep20. Replication protein binds to repetitive sequence...
in the origin of replication. Bioinformatic analysis, we confirmed the presence of motif with four intermittent alpha-helices. We prepare some mutants of HTH region and identified DNA-binding site.

References

**SW06.S25–61**

Glucose and intracellular peptides metabolism alteration in neurolysin knockout mice

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Neurolysin (EC 3.4.24.16) is a metallopeptidase widely distributed in the cytoplasm of mammalian tissues. In vitro, neurolysin can hydrolyze a number of bioactive peptides containing from 5 to 17 amino acids including neurotensin, bradykinin and several opioid peptides. Additionally, studies with the angiotensin-converting enzyme (ACE) transgenic mice containing 1, 2 or 3 copies of ACE gene and rats fed a high-caloric Western diet indicated the influence of intracellular neurolysin in metabolic homeostasis. In this report, for the first time, the production and initial phenotypic characterization of the neurolysin knockout mice (Nln<sup>−/−</sup>) is presented. Nln<sup>−/−</sup> mice are viable and have a normal reproduction and development. Nln<sup>−/−</sup> had increased glucose tolerance, insulin sensitivity and gluconeogenesis and are less tolerant to endurance exercise and shown a remodeling of fibers composition in skeletal muscle. In addition, Nln<sup>−/−</sup> have differences in specific intracellular peptides identified from skeletal muscle, liver and adipose tissue that could be related to the observed phenotype, corroborating previous suggestions that neurolysin play a role in physiological homeostasis. Moreover, it is exciting to suggest that intracellular peptide metabolism is contributing to the physiological regulation of glucose uptake, insulin signaling and exercise performance in mice. Financial support: FAPESP, CNPq, CAPES, Pró-reitoria de Pesquisa da USP (NAPPS).

**SW06.S25–62**

The thioacetamide-induced liver fibrosis in the murine animal model is associated with reduction in expression of glucose-regulated protein 78 (GRP78)

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**Novel Aspect:** Prior to the development of cirrhosis and cancer in liver, hepatic fibrosis usually has been detected in patients and it is the crucial stage for deteriorating liver disease, if without a good therapeutic management. Therefore, to define and detect the early biomarkers of liver fibrosis is very important issues for preventing form more severe forms of hepatic diseases such as cirrhosis and cancer. Several factors including viral infections, drug abuse and alcohol intoxication can contribute to liver fibrosis in human. In order to unbrand this mechanism and evaluate the therapeutic efficacy by liver fibrosis, the animal model has been created by injection of thioacetamide (TAA) to Wistar rats, leading to the development hepatic fibrosis in animals. TAA is a hepatic toxin can cause the liver fibrosis in murine, but the detail mechanism remains unclear. In this study, we will like to report our finding in uncovering the potential mechanism causing hepatic fibrosis in this TAA-induce rats using the tools including the matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF MS) and the two-dimensional differential gel electrophoresis (2D-DIGE).

**Methods:** Wistar rats were used for generating animals with diseases of AFL, non-AFL (NAFL), and liver fibrosis. AFL rats were orally fed 5 ml of a 36% alcohol solution for 4 weeks (6 g/kg/day). For rats with NAFL, animals were fed with diet containing 60% fructose or 45% fat for 12 weeks. Drinking water containing 0.04% thioacetamide (TAA) was supplied to animals for 12 weeks to generate the liver-fibrosis of rats. The sera from patients with non-alcoholic steatohepatitis or chronic hepatitis C virus (HCV) infection were collected and compared. To differentiate and validate the protein expression level, the biopsy specimen samples were characterized using MALDI-TOF MS, 2D-DIGE and western blotting.

**Preliminary Data:** In this study, we analyzed and compared to the clinical characteristics of non-alcoholic steatohepatitis (NASH) and HCV-infected fibrosis in animal livers. To identify the potential the marker proteins of liver fibrosis in TAA-induced rats, 2D-DIGE was performed and then the gel samples derived from healthy control liver specimen and animals with exposure to TAA were measured and compared. The results demonstrated that the histological activity index (HAI) was higher in animals with liver fibrosis than that in the NASH animals. Moreover, the combined data from 2D-DIGE and MALDI-TOF MS revealed the decreased expression level of glucose regulated protein (GRP) 78 in the fibrotic liver tissues of animals fed with food containing TAA, however, a significantly increased expression level of GRP78 was measured in AFL or NAFL animals. Furthermore, results from the TUNEL assay revealed a higher apoptotic cell numbers in the liver of TAA-fed rats, compared to that in healthy control, AFL, or NAFL. This indicated that a reduction in Grp78 protein expression may lead to ER-mediated apoptosis. Beside a reduction of GRP78 expression, and increased apoptotic cell numbers, we found that TAA also increased the production of reactive oxygen species (ROS). In conclusion, we demonstrated that GRP78 played a crucial role in maintaining ER physiological functions and the deficient in GRP78 may lead to apoptosis as well as trigger the onset program of liver fibrosis in TAA-treated rats.

**Keywords:** hepatic fibrosis, thioacetamide (TAA), Glucose-regulated protein 78 (GRP78), MALDI-TOF MS, 2D-DIGE.

**SW06.S25–63**

Excretory/secretory proteins of liver fluke *Opisthorchis felineus*

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*Opisthorchis felineus* (Siberian liver fluke) is neglected liver fluke causative agent of opisthorchiasis. The world’s largest focus of this disease is the Ob-Irtysh river basin in Russia.
The parasites inhabit the liver bile ducts and gallbladder of the definitive host, live a long time and can cause cholangitis, obstructive jaundice, hepatomegaly, periductal fibrosis, cholecystitis, cholangitis and probable can lead to development of bile duct cancer. Apart from mechanical damage and inflammatory response of host, factors secreted by *O. felineus* (secretory/secretory-ES) product and proteins exposed on tegument also play an important role in the former bile duct changes (host-parasite interplay) as well as liver fluke survival.

In the given study we performed a proteomic identification of the ES and tegument proteins from the adult worms of *O. felineus*. To identify complex mixture parasite proteins LTQ-FT-ICR were employed. The ES product contains 34 proteins with valid score such as seven isoforms of cystein peptidase cathepsin which were likely originating from the opisthorchis gut and also unambiguously identified 19 tegument proteins. Also *O. felineus* ES and tegument proteins included a number of parasite protective enzymes such as peroxiredoxin, enolase, and glutathione-S transferases and fatty-acid-binding protein and others. They may be the central players in the protection of *O. felineus* from the hostile environment. The comparison with the previous studies on the other liver flukes indicates that these enzymes are common to other parasite ES proteins and suggests the survival mechanisms may be conserved across different parasites. In addition our attention has been attracted to the fact that, defense proteins amounted about 50% from all proteins contained in the ES product and there were a lot of non-secreted proteins and cytoskeletal the ones (i.e. actin, tubulin, paramiosin) in the ES proteins.

The given study represents the first attempt at profiling *O. felineus* ES and tegument proteins provides an insight into host-parasite interactions and establishes a resource for the development of diagnostic and vaccine targets for the control of opisthorchiasis.

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**SW06.S25–64**

**Converting a plant defense peptide into a potassium channel blocker**

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It is well known that in proteins the same fold does not imply the same function. One good example is the potassium channel blocker charybdotoxin and invertebrate defensins that share a common fold but perform diverse functions. The α-hairpinin fold (two antiparallel α-helices stabilized by two disulfide bonds) may represent even a better case. It is shared by molecules with quite different functions: the plant antifungal peptide EcAMP1 from *Echinocloa crus-galli*, trypsin inhibitors BWI-2c from *Fagopyrum esculentum* and VhTI from *Veronica hederifolia*, ribosome-inactivating peptide luffin P1 from *Luffa aegyptiaca*, potassium channel blockers α-hefutoxin-1 from *Heterometrus fulvipes* and OmtX1–3 from *Opisthacanthus madagascariensis* and some others.

In this work we attempt engineering novel function in an α-hairpinin.

Tk-AMP-X2 is a potent antifungal peptide produced by the wheat *Triticum kiharae*. Its 3D structure was determined by NMR spectroscopy and shown to adopt the α-hairpinin fold. The critical dyad (one lysine and one tyrosine residue) essential for α-hefutoxin-1 function as a potassium channel blocker was then introduced onto the Tk-AMP-X2 scaffold. The mutant peptide was named Tk-hefu and produced recombinantly in *Escherichia coli* with a yield of ~4 mg per 1 l of bacterial culture. Both parent and mutant peptides were then tested for potassium channel blocking activity. Voltage-gated potassium channels K1,1.1, 1.2 and 1.3 were expressed in *Xenopus laevis* oocytes and corresponding currents were measured using the two-electrode voltage clamp technique. While the parent peptide did not show any activity, Tk-hefu blocked K1.3 (IC50 ~15 μM) with even greater potency than α-hefutoxin-1 (IC50 ~40 μM). Our results demonstrate the utility of the α-hairpinin fold for functional engineering.

This work was supported by the Ministry of Education and Science of the Russian Federation (contract no. 8794), the Russian Foundation for Basic Research (grant nos. 11-04-00190 and 12-04-33151), and the Molecular and Cell Biology Program of the Russian Academy of Sciences.

**SW06.S25–65**

**Peptidome profiling of induced sputum by mesoporous silica beads and MALDI-TOF MS for biomarker discovery of Asthma and COPD**

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Induced sputum (IS) is an inestimable source of inflammatory mediators. Given its high protein, cellular and microbiological components, IS is used for monitoring severity, exacerbation and progression of lung pathologies [1]. Among lung diseases, asthma and COPD are particularly relevant due to their increasing diffusion. However, they are not always clearly distinguishable with the current clinical and functional assessments. Therefore, a novel analytical method able to differentiate these two respiratory disorders is still an unmet medical need. As a part of an ongoing study aimed to identify differentially expressed peptidic patterns in IS of COPD and asthma patients, we tested the use of mesoporous silica beads (MSB) for processing IS. Then, MALDI-TOF MS was used for differential peptide display in the range from 800 to 10 000 Da using CHCA as matrix. Six *m/z* peaks emerged as potential diagnostic peptidic patterns able to differentiate these inflammatory airway diseases in the sputome range, among which Human α-defensins [2,3]. We are now optimizing the methodology to find novel important specific protein patterns able to differentiate asthma from COPD extending the previous range of MW from 4500 to 20 000. To this end, we processed IS with MSB and enriched fractions were then analyzed by MALDI-TOF MS by using sinapinic acid as matrix of choice. Although the number of peaks in the unprocessed IS samples was higher in comparison to the MSB processed samples, the use of MSB allowed the detection of novel peaks which were totally absent in the MALDI spectra of unprocessed samples. Additionally, a significant increase of S/N ratio was observed for more than the 30% of the peaks common to both samples. Interestingly, the range between 10 and 14 kDa of MALDI-TOF read-outs of MSB processed IS samples evidenced a distinctive fingerprints common to 80% of asthma patients and a typical signature common to 90% of COPD patients. These findings may contribute to delineate a high-throughput screening MS-based platform for monitoring key peptidic-signatures for asthma and COPD diseases in IS samples.

**Reference**

SW06.S25–66
Interphase chromatin defined probabilistically using biological perturbation proteomics
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Specialised compartments play a fundamental role in many cellular processes. The composition and plasticity of such structures can be studied by proteomic approaches. Early efforts have analysed organelles following isolation or quantitative enrichment. A second generation of strategies has studied subcellular compartments based on co-fractionation with marker proteins on density gradients. Defining organelles in this way critically depends on biochemical procedures and inherently introduces a series of purification artefacts. We have recently circumvented some of these by an approach called multiclassifier combinatorial proteomics (MCCP). Here we depart from the idea of definite component lists for organelles and the possibility of defining these biochemically. We propose a novel concept that centres on probabilities and dynamic biological behaviour. To define human interphase chromatin, a biochemically intractable structure, we use proteomics to follow biological co-behaviour of proteins with known chromatin players over a spectrum of different physiological conditions (e.g. cell types and drug treatments). These experiments are combined using MCCP. On the basis of known chromatin proteins, we then derive chromatin ‘integrated compartment probabilities’ (ICPs), the probability for a protein to have a chromatin-based function. We provide chromatin ICPs for 7600 human proteins, including 1800 previously uncharacterised proteins, helping researchers to focus large datasets on chromatin-based biological processes. Our approach aims at assessing protein function and overall is more successful at discriminating chromatin from non-chromatin proteins than biochemical experiments alone. ICPs are large-scale data-driven, stand-alone parameters that could complement annotation databases, such as Gene Ontology with an urgently needed quantitative dimension, paying reference to dynamic composition of organelles.

SW06.S25–67
Cell penetrating peptides (CPP) as the intracell delivery system for anticancer agents
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Although Chemotherapy has been responsible for curing many people of cancer in the first decade of the 21st century, there still remain a large number of patients whose tumors still are not curable. There has been substantial investment in researching the mechanisms used by normal eukaryotic cells to control progression through the cell cycle in the hope that this would lead to an understanding of how cancer arises and suggest possible targets for cancer therapy.

During carcinogenesis, it is currently thought that normal cells become immortalised as a consequence of disruption of the positive and negative cell signalling pathways and the cell cycle control mechanisms, for example, amplification and overexpression of cyclins and CDKs. Amplification and overexpression of cyclin D protein occurs in many human tumors.

We investigate the ability to direct the import of therapeutic agents into cells and target them to specific organelles with specific cell penetrating peptides (CPP). Transduction of protein of interest into cells by the help of peptides can serve as a good alternative to other mechanism of protein transport and protein expression. So we can conclude CPP only possible way for effective intracellular delivery this new therapeutic agents.

We investigate transport properties a wide range of peptides with different sequences and position of leading CPP in different part of chimeric peptide molecules. Based on our results we proposed new mathematical model for intersection of the cell membrane by CPP.

SW06.S25–68
Nuclear protein complexes of actin-binding protein alpha-actinin 4
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Nonmuscular actin-binding protein α-actinin-4 is located in the cytoplasm and in the nucleus. However, nuclear functions of α-actinin-4 are still not clear. In this study, we analyzed composition of nuclear protein complexes of α-actinin-4 in A431 cells. Using 2D electrophoresis, we have determined that about 50 different proteins may be associated with nuclear α-actinin-4. Using MALDI-TOF mass-spectrometry, we identified major proteins of these complexes: α- and β-tubulins; β-actin, ribonucleoproteins A2/B1, A1, K/L. Detection of ribonucleoproteins in association with α-actinin-4 suggest that it is involved in regulation of RNA metabolism. It was shown, that α-actinin-4 is involved in protein complexes of transcription factor NF-kappaB in nucleus of A431 cell and may be participate in regulation of its activity. A great deal of proteins with different functions, associated with α-actinin-4 in nucleus is evidence of existence different protein complexes of this protein in nucleus. Using native electrophoresis and gel filtration we found that there are at least two protein complexes of α-actinin-4 with masses 400–700 kDa in nucleus. These data suggest that there are several protein complexes of α-actinin-4 in nucleus, which may carry out different functions in regulation of gene expression and RNA metabolism.

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SW06.S25–69
Mechanistic aspects of translational inhibitor microcin C maturation
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Microcin C (McC) is an antibacterial agent produced by Escherichia coli that inhibits the protein synthesis. McC is formed by extensive post-translational modification of ribosomally synthesized heptapeptide precursor which results in the AMP attachment to the precursor’s C-terminal aspartate through an N-acyl phosphoramidate bond and additional modification of attached AMP by the 3-aminopropyl moiety. McC enters the cytoplasm of the sensitive cells through the specific oligopeptide transporter. Inside the cytoplasm McC undergoes processing by non-specific aminopeptidases. The product of such processing is a structural analogue of the aspartyl-adenylate – an intermediate of the aspartyl-tRNA synthesis. The processed McC inhibits aspartyl-tRNA synthetase, which in turn leads to inhibition of translation.

The genes responsible for McC biosynthesis, transport, and self-immunity of McC-producing cell are organized in the mccaBCDEF cluster. McC peptide precursor is encoded by 21-bp long mccA gene. Products of mccB, mccD, and mccE genes
are responsible for the post-translational modifications. MccC gene encodes McC export pump and the products of mceE and mceF genes provide resistance to McC.

MccB catalyzes AMP attachment to the McC peptide precursor whereas MccD and MccE are required for the 3-aminopropyl modification of the phosphate group. It is known that McC precursor without 3-aminopropyl group possess an antibacterial activity too which is however tenfold lower than that of the ‘mature’ McC with 3-aminopropyl group.

We have shown that aminopropilation is carried out via two step mechanism. Firstly MccD catalyzes attachment of the 3-carboxy-3-aminopropyl group to the phosphate of the McC precursor. Subsequently the attached moiety undergoes decarboxylation by the action of MccE N-terminal domain (MccE-NTD). It was demonstrated that MccD uses S-adenosyl methionine as a donor for the 3-carboxy-3-aminopropyl group whereas MccE-NTD requires pyridoxal-phosphate cofactor for catalysis. It was also shown that MccD and MccE proteins gain no sequence specificity for the peptide part of the McC. Based on our experimental data we also suggest that the McC precursor with the 3-carboxy-3-aminopropyl group is not exported by the MccC pump.

SW06.S25–70
The CD36 scavenger receptor in the phagocytic engulfment of oxLDL particles in human U937 macrophages
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Atherosclerosis is a leading cause of morbidity and mortality in developed countries. Atherosclerosis is an inflammatory disease in response to the buildup of fatty deposits within the intima of the walls of the artery. Macrophages become activated upon binding to oxidized low density lipoprotein (oxLDL) deposited on the walls of the arteries resulting in the production of free radicals inducing oxidative stress, the formation of foam cells and atherosclerosis. CD36, a member of the class B scavenger receptor family, is known to play a key role in the uptake of oxLDL by macrophages. In this study, U937 cells were treated with PMA to induce monocytic differentiation. Monocyte derived macrophages were stimulated with microbeads coated with oxLDL or immunoglobulin (IgG) ligands to examine the oxLDL internalization pathway by mass spectrometry and confocal microscopy. Multiple peptides were correlated to CD36 like molecules in the oxLDL and IgG receptor complexes as determined by nano LC-ESI-MS/MS and confirmed by confocal microscopy and Western blots using anti CD36 IgA, IgM and IgG probes. Immunostaining with, versus without, detergent revealed that most of the CD36 signal was ectopic and thus associated with the external leaflet of the plasma membrane. Silencing RNA against CD36 inhibited the uptake of oxLDL beads by about 40% but did not inhibit IgG bead engulfment. Hence it may be possible to inhibit the phagocytic engulfment of oxLDL by macrophages without preventing bacterial clearance.

SW06.S25–71
Analysis of the secretome of human granulosa cells after hormonal stimulation by various gonadotrophins
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Introduction: The major functions of granulosa cells (GCs) include the production of steroids, as well as a myriad of growth factors to interact with the oocyte during its development within the ovarian follicle. After conception the granulosa cells build together with invading endothelial cells the corpus luteum. Insufficiencies in granulosa cell function leads to many pathologies including infertility, defects in oocyte development and abortion. Many of these pathologies are caused by hormonal imbalances acting directly on granulosa cells. Progress in the emerging field of proteomics allows analyzing proteins derived from small samples. Here we report about our efforts to analyze the secretome of human granulosa cells after stimulation with various gonadotropins used in infertility treatment.

Material and Method: The secretome of human granulosa cells and the granulosa carcinoma cell line KGN under gonadotrophic hormonal stimulation were compared versus controls. Protein precipitation of samples was performed by Methanol/Chloroform following trypsin digestion. Separation of digested proteins was performed by liquid chromatography and two types of mass spectrometers: the ion trap for peptide mapping and the qToF (time-of-flight) mass spectrometer for characterization and quantitation of selected proteins. The database search is performed with the commercially available MASCOT software from Matrix Science and the data are further filtered and analyzed by applying Scaffold.

Results: Using a probability score of 95% we detected around 80 different granulosa cell secreted proteins. After hormonal stimulation the secreted protein pattern changes. Beside known proteins like TIMP-1 some previously unknown proteins were detected only in the supernatant of hormonal stimulated granulosa cells. One of these proteins belongs to the S100 family of low molecular weight proteins characterized by calcium binding sites of the helix-loop-helix EF-hand conformation. Although still poorly characterized, secreted proteins of this family were described to be involved in regulation of protein phosphorylation, calcium homeostasis, cell growth and differentiation, and the inflammatory response.

Conclusion: The proteome based analyses of secreted proteins provide a useful tool to understand the processes during hormonal stimulation in human granulosa cells. It may help to define granulosa cell derived distinct protein profiles causative for human pathologies with regard to infertility and reproduction.

SW06.S25–72
Analyzing the preimplantation secretome of human embryos
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To elevate pregnancy rates after IVF new technical and methodical more efficient approaches beneath PGS are needed. Currently,
the ‘quality’ of fertilized oocytes embryos is based on morphological criteria and subjective judgment of the physician performing the procedure. Our hypothesis is that proteins secreted from developing embryos differ depending on the ‘health’ of the particular embryo.

One new approach could be the analysis of the embryos’ proteome during the IVF process. To understand this procedure, it is important to know that, during incubation and before implantation, embryos must be transferred to a new culture medium every day or at least when becoming an embryoblast at day 4, respectively. After retrieval of the embryo and the implantation, the remaining medium is normally discarded. However, we use this medium for analysis of secreted proteins that were secreted by the embryo during the previous incubation process.

Fertilized oocytes are cultivated in media containing high percentage of serum albumin which, beside the low amount of secreted proteins and the low sample volume, is the main obstacle for analysis of proteins secreted from developing embryos. One of the major tasks is to remove the albumin and analyze the remaining protein pool.

The combination of online and offline depletion and digestion procedures resulted with identifications of proteins revealing important information on embryo communication with its environment and possible pregnancy development.

**SW06.S25–73**

**Tetraspanin proteins in the IgG-Fc receptor complex from human U937 macrophages**

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The Fc receptors belong to the immunoglobulin superfamily. The ligand of Fc gamma receptor is the immunoglobulin IgG, which triggers the engulfment of foreign molecules coated by antibodies referred to as phagocytosis. Tetraspanins may act as ‘molecular facilitators,’ grouping specific cell-surface proteins and thus increasing the formation and stability of functional signaling complexes. The Fc receptor complexes were isolated using live-cell affinity receptor chromatography (LARC). The Fc receptor complex bound to its cognate ligand IgG presented on 2 μm polystyrene beads. The Fc receptor was captured from the surface of live human macrophage U937 cells using IgG-coated micro beads and analyzed by liquid chromatography and tandem mass spectrometry alongside several controls. The controls used in this experiment include IgG-coated beads incubated with crude cell lysates or uncoated beads mixed with crude extract or experimental medium as blanks. The ligand beads were presented to the live cells and incubated from 0 to 4 h, the cells were quenched on ice, homogenized with a French press, the receptor complexes were isolated by sucrose gradient in an ultracentrifuge, the beads were washed, eluted with salt or acetonitrile and then digested with trypsin alongside the control beads. The peptides were identified using LC-ESI MS/MS with a linear ion trap followed by MS/MS correlation by the SEQUEST algorithm where the results from the IgG versus control beads were stored in an SQL database. A number of Tetraspanin isoforms were found to be specific to the Fc receptor complex. Silencing RNA and quantitative assays of phagocytosis using laser scanning confocal microscopy were used to assess the importance of the specific Tetraspanin isoforms in Fc receptor mediated phagocytosis.

**SW06.S25–74**

**The peptides of normal human blood plasma**

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The solid phase extract of endogenous peptides from human plasma by mixtures of aqueous and organic solvents was previously compared to albumin isolation, ion exchange chromatography, ultra-filtration, solid phase extraction with C18, organic precipitation with a variety of solvents, and the combinations. In this experiment, all polyepitides were precipitated in 90% acetonitrile followed by the progressive extraction of low molecular mass peptides in mixtures of organic solvents and water that dissolved peptides while maintaining proteins in the solid phase prior to decanting, drying and preparative collection of the peptides over C18 reversed phase. Peptides were analyzed by analytical C18 liquid chromatography with nano electrospray ionization followed by tandem mass spectrometry (LC-ESI-MS/MS). The parent molecules of factors known to have low nano molar or pico molar concentrations were clearly observed. Peptides derived from signal transduction molecules including kinases, G-proteins and their adapter and regulatory proteins were well observed. Furthermore, peptides from the parent molecules of the ligands, receptors and/or binding proteins of interleukins, chemokines, cytokines, tumor necrosis factor and growth factors were observed. The parent ion and fragment ion intensity values from all of the plasma peptides and their MS/MS fragments were recorded in a structure query language (SQL) database. Novel blood peptides proteins associated with important cellular functions and biological processes were recorded by tandem mass spectrometry.

**SW06.S25–75**

**The presence of integrins specifically associated with activated FC receptor complexes from human U937 macrophages**

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Certain integrin isoforms were shown to be associated with the Fc receptor complex by ligand affinity chromatography in live cells and mass spectrometry. The Fc receptor complex was captured using 2 μm polystyrene beads coated in Immunoglobulin G (IgG) and incubated with live human U937 macrophages. The cells were disrupted using a French press and the Fc-IgG-beads were isolated by ultracentrifugation over a sucrose gradient. The same ligand coated beads were incubated with crude extracts for classical ligand affinity chromatography. Uncoated beads incubated with crude extracts or used experimental medium served as controls for non-specific binding. The receptor complexes were fractionated with increasing salt and acetonitrile in step gradients and digested with trypsin. Insoluble proteins were digested directly on the beads with and without organic solvents. The peptides were collected over C18 preparative chromatography prior to analytical separation over C18 HPLC and tandem mass spectrometry (LC-ESI-MS/MS). The resulting peptides were identified using the SEQUEST algorithm. The resulting peptide and protein identifications with the associated MS, and MS/MS spectra, including the ion m/z and intensity data, was subsequently stored in an SQL database. Certain integrins were found to be specific to the Fc receptor complex. The functional role of the specifically detected integrins was examined by silencing RNA and quantitative phagocytosis assays using laser confocal microscopy. There was a functional role for some integrin isoforms in Fc mediated phagocytosis.
SW06.S25–76
Proteomic analysis of epileptic human brain – ‘alcoholic’ mobile phase detects more proteins
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Separation of tryptic peptides by reversed-phase (nano) high-performance liquid chromatography (RP-HPLC) commonly mobile phase systems containing acetonitrile as modifier organic are used. Following the HPLC separation eluted peptides are detected using in-line electrospray ionization tandem mass spectrometry (ESI-MS/MS). Now a new approach using HPLC eluents containing alcohols such as methanol and 2-propanol instead of acetonitrile is described. The percentage of alcohol in the mobile phase was varied and the results were compared to separation of the same tryptic peptides samples under (otherwise) default separation conditions (organic mobile phase containing acetonitrile). We will use peptides obtained from brain tissue biopsies (amygdalohippocampectomy) and as biological sample in order to show the benefits of the newly developed separation methods.

Methods: The nano HPLC system used in all experiments was an UltiMate 3000 RSLC HPLC system (Dionex) coupled to Maxis 3G-qToF MS (Bruker). All samples were separated using Acclaim PepMap C18 column; 100 Å pore size; 3 μm particle size; 0.075 mm ID x 250 mm. Both columns were operated at 45°C in a column oven. For test purpose, 500 fmol of the BSA tryptic digest was injected. Subsequently, the separation results for the biological sample (digested proteins from human epileptic brain) were compared for the default mobile phase and the mobile phases containing alcohol.

Preliminary Data: The increase of the alcohol part in mobile phase led to significant selective changes in peptide retention times on to the reversed-phase system and in intensity of the MS/MS spectra. These changes are the consequence of different physico-chemical conditions in retention such as change of their overall charge, polarity and relative hydrophobicity of peptides in a solvent of different solvation properties. Now, the peptides eluted uniformly over the complete gradient window without grouping in certain areas which enables generally better separation and better MS detection.

As a further consequence of these changes, different selectivity of a separation column with different peak capacity was observed. The use of the new ‘alcoholic’ mobile phase enabled detection of higher amounts of peptides and proteins.

A significant increase in identifications of unique peptides and a better sequence coverage for identified proteins were observed with the ‘alcoholic’ mobile phases.

SW06.S25–77
Secretome profiling of senescent mesenchymal stem cells (MSC) by high resolution LC-MS analysis
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Cellular senescence is the permanent arrest of cell cycle that has been thought to be physiologically related to aging and aging-associated diseases. Senescence is also recognized as a mechanism that limits the regenerative potential of stem cells and protects cells against cancer development. The senescence program is realized through autocrine/paracrine pathways based on the activation of a peculiar senescence-associated secretory phenotype (SASP). We demonstrated that conditioned medium of senescent mesenchymal stem cells (MSC) is able to induce a full senescence response in young cells as confirmed by a decrease in cell proliferation and the increase in senescence-associated β-galactosidase activity. Using a comparative and functional proteomic approach we characterized the factors secreted by senescent MSC with the aim to define a hallmark of stem cells SASP. We identified insulin-like growth factor binding proteins 4 and 7 (IGFBP4 and 7) as key factors of senescent MSC conditioned medium for triggering senescence phenomena in young MSC. These pro-senescent paracrine effects were reversed by either single and/or simultaneous immunodepletion of IGFBP4/7 from senescent conditioned media. Moreover, the blocking of IGFBP4/7 also reduced apoptosis and promoted cell growth, suggesting that they may have a pleiotropic effect on MSC biology. Collectively, these results identify novel components regulating MSC cellular senescence of potential importance for regenerative medicine and cancer therapy.

SW06.S26 Metabolism of Marine Organisms: Structure and Activities (VI-S26)

SW06.S26–1
Drug and gene delivery systems based on the chemistry of a marine polysaccharide
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Chitosan (CS) is a polysaccharide of marine origin very much used in biomedical applications due to its biocompatibility, low
toxicity, mucoadhesiveness and ability to form nanoparticles. It is a partially N-acetylated poly(glucosamine) and its a properties are highly dependant on the acetylation degree, and M.W. (1).

The general methodology for the chemical modification and characterisation of CS and CS-g-copolymers carrying different proportions of PEG chains (2), fluorescent labels, drugs, and antibodies, as well as their transformation into nanoparticles (3) as transport systems, will be illustrated with examples of delivery of drugs (4) and genes (5).

Two general schemes will be presented where a) the incorporation of the fluorescent labels, drugs, and antibodies to the biopolymer is carried out before the formation of the nanoparticle or, b) the particle is initially formed from the starting CS copolymers and then, its surface is decorated with labels and antibodies, using Click chemistry (6). Special attention will be paid to practical aspects of the reactions with polymers, their monitoring by NMR and to efficient ways to obtain good NMR spectra of the modified polymers and their mixtures in short times (7).

References


SW06.S26–3
New immunomodulators from sea cucumbers: molecular mechanisms of action

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The holothuroid triterpene glycosides at micromolar concentrations exhibit strong cytotoxic activities against different cell types. The basis of the glycoside cytotoxicity is their ability to interact with cellular biomembranes and form nonselective ion-conducting complexes with 5(6)-unsaturated sterols. As result the disruption of ion homeostasis and osmolarity followed by lysis and cell death take a place. On the other hand, some glycosides demonstrate a pronounced immunostimulatory effect at sub-toxic nanomolar concentrations. Present work demonstrates that incubation of immune cells with the cucumarioside A2-2 from the Far Eastern sea cucumber Cucumaria japonica results in their activation. At nanomolar concentrations cucumarioside A2-2 is associated only with high affinity receptors. Using confocal microscopy, flow cytometry, Ca2+ imaging and electrophysiology (patch-clamp) it was found by us that the most likely membrane targets of this glycoside can be purinergic receptors of P2X family (predominantly P2X1 and P2X4). Structural models of A2-2–related to pain. Perception of pain depends on special receptors and pathways. It is known that TRPV, ASIC and P2X receptors play an important role in the processes of generation, transduction and processing of pain signals. The guided search of novel modulators of these receptors is an indispensable way for development of new medical products. Venoms of marine organisms such as snakes and sea anemones, and terrestrial animals such as spiders are considered as peculiar naturally edited combinatorial libraries of polypeptide molecules. Venom components are directed against a wide variety of membrane targets such as ionotropic receptors and channels. These components can discriminate between closely related cellular targets, which makes them attractive for scientific use and drug development. Peptides have several advantages as therapeutics in terms of the high degree of specificity and efficacy. Analgesic peptides named APHC1 and APHC3 were isolated from the extract of sea anemone Heteractis crispa. They consist of 56 residues and assume the Kunitz-type fold. Both compounds partially block the capsaicin-induced response of TRPV1, but none of them influence on thermal activation. We characterized these peptides as potent TRPV1 modulators and compared their pharmacology in various in vitro and in vivo models. A NOVEL PEPTIDE UGR 9A-1 WAS FOUND IN THE VENOM OF the sea anemone Urticina grebelnyi. IT CONSISTS OF 29 RESIDUES cross-linked by two disulfide bridges. UGR 9-1 PRODUCES a reversible inhibition effect on both the transient and sustained current of human ASIC3 channels. It significantly reverses inflammatory and acid-induced pain in mice. NMR spectroscopy found that Ugr 9-1 has AN UNCOMMON β-hairpin spatial structure. A P2X3 receptor modulator was identified in the venom of the wolf spider Geolycosa sp. Its peptide component named purotoxin-1 (PT1) contains 35 residues and conforms to the ICK fold. PT1 dramatically slows down the removal of desensitization of P2X3 receptors and demonstrates potent antiinociceptive properties in animal models. Recent results on the mode of action and analgesic properties of the peptides will be presented.

SW06.S26–2
Analgesic peptides from animal venoms

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Chronic pain diseases great number of people worldwide. An absolute majority of all cases of people seeking medical help is

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mP2X1 and nP2X4 complexes generated by in silico modeling disclosed that cucumarioside A₁-2 and ATP binding sites are localized at different areas of extracellular receptor domain. Molecular Dynamics simulations results revealed that cucumarioside A₁-2 action on the receptor turn those binding site residues connected through β-strands by H-bonds network. These findings and experimental data suggest that cucumarioside A₁-2 can act as an allosteric regulator which able to withdraw purine receptor inactivation by extracellular ATP and provide a recovery of Ca²⁺ conductivity of macrophage membrane. Such type of glycose interaction with receptors may trigger an activation of Ca²⁺-signaling pathway that initiates the amplification of expression of certain intracellular target proteins involved in key stages of immune cell physiology. This leads to increasing in cell adhesion, spreading, motility and proliferation, ROS formation, rise in lysosomal activity, phagocytosis, elevated synthesis of some cytokines and pathogenic microorganism killing. Ultimately, an activation of cellular immunity and magnification of the organism resistance to various opportunistic infections is appeared under glycose action, as it was recently demonstrated by us experimentally.

**SW06.S26-4**

**Fucoidan exerts immunogenic anti-tumor effects through scavenger receptor type A**

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Fucoidan, a sulfated polysaccharide in brown seaweed, has various biological activities, including activation of immune cells and antitumor activity. However, the molecular mechanism by which fucoidan regulates activity of immune cells has not been clarified. In this study, the effects of fucoidan on the maturation and migration of dendritic cells (DCs), which are required for effective immunity in cancer patients, were investigated. In murine cancer models, intravenous adoptive transfer of bone marrow-derived DCs (BM-DCs) in combination with fucoidan achieved tumor growth inhibition in tumor-bearing mice. The systemic administration of fucoidan induced maturation of adoptively transferred BM-DCs and DCs in secondary lymphoid tissues of mice. However, scavenger receptor type A (SR-A)-depleted DCs and splenic DCs in SR-A-deficient mice failed to be matured by injection of fucoidan. The lymph node-homing chemokine receptor, CCR7 was expressed at significantly higher levels on administered BM-DCs and splenic DCs in fucoidan-injected mice than LPS-injected mice. Fucoidan efficiently induced migration of labeled wild type BM-DCs but not SR-A-depleted BM-DCs to spleen and tumor tissues. In addition, less number of wild type BM-DCs were migrated to lymphoid tissues in SR-A-deficient mice than in controls with injection of fucoidan. These results suggest that combined fucoidan and DC therapy exerts immunogenic response against cancer cells through SR-A-dependent trafficking and maturation of DCs to lymphoid tissues.

**SW06.S26-5**

**New natural products from sponges: structures and properties**

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Sponges are widely recognized as the most prolific source of structurally unique and biologically active marine natural products. They are also abundant benthic invertebrates of tropical, temperate and boreal waters and play an important role in the corresponding marine ecosystems. Our studies have been focused on the isolation and structure determination of novel compounds from these animals. By now, we have isolated, established chemical structures, and studied biological activities of about 200 compounds from numerous species of marine sponges. These substances belong to a wide variety of biogenetic classes such as two-headed sphingolipids, isoprenoid sulfates, pentacyclic, bicyclic and monouncyclic guanidine alkaloids, etc. Monanchocidins¹,² and monanchomycalins³ isolated from the Far-Eastern sponge *Monanchora pulchra* are noticeable examples of recently discovered novel marine bioactive molecules. These compounds possess powerful antileukemic properties and present interest from the point of view of their biosynthesis. The first marine non-peptide inhibitors of TRPV-1 channels pulchransins A-C also were isolated by us from *M. pulchra.*⁴

We will discuss the diversity of unusual chemical metabolites in sponges from the North-Western Pacific, the details of their isolation and structure elucidation, and provide insights into molecular mechanisms underlying their biological activities.

**References**


**SW06.S26-6**

**Effect of dimethylsulfoxide on redox potential and hydrogen photoproduction by Rhodobacter sphaeroides**

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Dimethylsulfoxide (DMSO) is a water-miscible solvent which has wide applications in biochemistry. DMSO affects on the growth and survival of the various bacteria [1,2]. In this study the effect of the various concentrations of DMSO on hydrogen (H₂) yield of purple bacterium *Rhodobacter sphaeroides* MDC6521 (from mineral springs in Armenia) and its correlation with redox potential were investigated. This study is important for understanding of mechanisms for biochemical effects of DMSO on bacterial growth and H₂ photoproduction. Low concentration of DMSO (1–5 mM) enhanced phototrophic growth of *R. sphaeroides*, whereas the high concentration (10–50 mM) suppressed the growth. Photosynthetic pigments have been found to be sensitive to the growth medium content. By addition of DMSO a decrease
in the level of photosynthetic pigments was observed. The redox potential can be considered as the important factor determining bacterial anaerobic growth, which is connected to decreasing of potential from positive to negative values. A relationship between the H$_2$ production and falling of redox potential was observed: the electron flow can be shifted toward the reduction of proton to H$_2$ under strong reducing conditions. The results point out the concentration dependent DMSO effect. In the presence of 1–5 mM DMSO redox potential, measured by platinum electrode, decreased up to ~700 mV, and a higher H$_2$ yield was detected. In the presence of 10 mM DMSO redox potential decreased up to ~465 mV and inhibition of H$_2$ yield (~6 fold) was observed. Redox potential was not changed much, and H$_2$ production was not observed by addition of 30–30 mM DMSO. Thus, DMSO in appropriate concentration might affects the synthesis of photosynthetic pigments, and the activity of nitrogenase, the key enzyme, involved in H$_2$ photoproduction by *R. sphaeroides*.

References


**SW06.S26–7**

Chemical and molecular approaches towards the biosynthesis of the modular cyclohexadepsipeptide anticancer agent homodolastatin 16

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Homodolastatin 16 (C$_{50}$H$_{72}$N$_{18}$O$_{19}$), a cyclohexadepsipeptide isolated from the Kenyan tropical marine cyanobacterium *Lyngbya majuscula* shows moderate activity against the esophageal cancer cell lines WHCO1 and WHCO6 with IC$_{50}$ values of 4.3 and 10.1 µg/ml respectively. Strikingly, however, synthetic modification results into the significantly more potent analogue dolastatin 16. Genome mining and bioinformatics strategies have been employed to search for the genes encoding nonribosomal peptide synthetase (NRPS) modular assembly of homodolastatin 16 in *Moorea producens* 3L genome. These strategies identified proline (Pro) and the SAM mediated (S-adenosyl methionine) isoleucine adenylation domains in the molecule as originating from the cyanobacterium but provided little clue on the open reading frame (orf) of conserved gene clusters of the compound and its biogenesis. Herein, we provide the theoretical framework for the biosynthesis of homodolastatin 16 that delineates it from other NRPS modular cyclodepsipeptides from *L. majuscula*. An unusual dophenvaline (Dpv) amino acid formed from methylation of an α-keto acid followed by transamination to β-methyl phenylalanine; together with the hybrid NRPS/PKS module condensing valine and methyl malonyl CoA leading to the formation of the dolamethyline (Dml) residue extension in the structure of homodolastatin 16 is the link towards identifying the gene cluster encoding for the molecule. The modular assembly also highlights the possible reversal of the peptide chain system and an epimerization domain for an L-Pro-oxo-L-Hiv intermediate that is key to the conversion of L-Hiv to D-Hiv. These approaches will aid in identifying gene clusters of homodolastatin 16. It is our aim to heterologously express this gene cluster in order to improve the fermentative production of this medicinally interesting anticancer agent and its analogues.

**SW06.S26–8**

Purification and characterization of a novel exo-cellulase produced by the deepest-sea amphipod ‘Hirondella gigas’

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In 1960, the bathyscaphe Trieste voyaged to the bottom of the Challenger Deep in the Mariana Trench, the deepest point in the ocean (10 994 m), where its crew discovered that some organisms could withstand extreme pressure$^1$. The amphipod *Hirondella gigas* is a resident of this deepest abyss. Amphipods are major inhabitants of the great depths, serving as scavengers. The ecology of such abyss creatures is still unknown and very mysterious. Half a century has passed since the discovery of *H. gigas*; however, there is relatively little information about this organism’s physiology and ecological interactions in the hadopelagic zone. How do they get enough nutrients? How do they adapt to such a harsh environment? Our first purpose is investigation of the nutrient uptake process of *H. gigas*. We captured an almost ‘pure’ culture of *Hirondella gigas* from the Challenger Deep. Then, we analysed whole-body extracts for digestive enzymes to understand this scavenger’s feeding behaviour.

We detected amylase, cellulase, mannanase, xylanase and α-glycosidase activities, which would be capable of digesting plant-derived polysaccharides. We focused on cellulase because of its products of glucose and cellobiose. Purified *H. gigas* cellulose converted cellulose to glucose and cellobiose at a remarkable molar ratio of 2:1 and produced glucose efficiently from dried woods at 35°C. Our study strongly suggests that during its evolution, *H. gigas* adapted to its extreme oligotrophic hadal oceanic environment by expression of enzymes capable of digesting sunken wooden debris.


**SW06.S26–9**

Mechanisms of anticancer action of the different alkaloids from marine organisms

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Drug discovery from marine organisms has enjoyed a renaissance in the last years. The marine world has become an important source of anticancer agents with novel mechanisms of action. The intensive studies have allowed to isolated, identified and synthesized the different of alkaloids from marine organisms, possessing high inhibiting activity in respect of varied line tumor cell and showing significant of anticancer effect in respect of different experimental human and animal tumor models, as well as having high-quality therapeutic potential when test in clinical conditions. Trabectidin (Yondelis; PharmaMar) become the first marine anti-cancer drug to be approved in European Union. In current work is made review up-to-date dates about plausible mechanisms of anticancer action of the different alkaloids from marine organisms, focusing in the imidazole alkaloid polycarpine from the ascidian *Polycarpa aurata* and its synthetic analogues; in pyridoindol alkaloid fasacyspin from the tropical sponges as selective inhibitor of cyclin-dependent kinases 4 (IC$_{50}$ = 0.4 µM), which play a key role in the cell cycle and is important anticancer drug targets; in pyrroloiminoquinone alkaloids of discorhabdins and makaluvamines from various marine sponges, which very effectively inhibited of growth of various tumor cell lines in vitro,
and/or are possessed of anticancer activities in vivo. It is possible that indicated above alkaloids from marine organisms will represent as ‘biochemical probes’ and valuable tools in treatment of oncological diseases.

SW06.S26–10
Mechanistic understanding of the bacterial bioluminescence
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Bioluminescence is the production of light by living organisms using enzyme-catalyzed reactions as a key factor to release the energy. The yellow-green light emitted by fireflies is a well-known example for bioluminescence. The bioluminescent reaction is catalyzed by luciferase, an enzyme employing FMN as a redox cofactor to drive the monooxygenation of the aldehyde substrate to the acid product. The free energy released during the oxidation of the aldehyde gives rise to an excited state FMN-4a-hydroxide, which in-turn serves as the luciferin in bacterial bioluminescence, as shown in the following reaction:

\[
\text{FMNH}_2 + O_2 + RCHO \rightarrow \text{FMN} + H_2O + RCOOH + \text{light} \ (\lambda = 490 \text{ nm})
\]

The genes for bacterial light production are present as an operon, luxCDABEG: luxA and luxB encode the subunits of luciferase; luxC, luxD, and luxE specify the enzymatic components of a fatty acid reductase complex necessary for synthesis and recycling of the aldehyde; and luxG encoding a flavin reductase. Many strains of Photobacterium also carry an extra gene, termed LuxF, having a lux operon gene order of luxFDBEG. Sequence similarity to luxF suggests that luxF has arisen by gene duplication, however, its role in bacterial bioluminescence is obscure especially because only free-living but not symbiotic photobacteria appear to exhibit the luxF insertion in their lux-operon. Its main function might be to bind the myristylated FMN (myrFMN), a possible side product of the luciferase reaction, which is believed to bind sufficiently tight in the active site of luciferase and thus would lead to inhibition of the bioluminescence reaction.

The generation of 6-((3'-R)-myristyl)FMN (myrFMN) in the genus photobacteria is a largely unexplored phenomenon in bacterial bioluminescence. In the present study, we investigated the occurrence of myrFMN in bioluminescent bacteria by exploiting the property of LuxF to bind to this modified form of FMN. We have developed a protocol to isolate and characterize myrFMN and initiated experiments to demonstrate its generation. In addition, we have generated knock-out strains to study the role of luxF in vivo.

SW06.S26–11
Influence of cucumarioside A2-2 upon multi-drug resistance of cancer cells
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It is known that one of the major causes of tumor cell drug resistance is decreased accumulation of cytotoxic agents in the cell. This so called multi-drug resistance (MDR) is associated with active transport and removal of substances by cell membrane drug pumps, like P-glycoprotein. Recently, it was found that the newly identified, immunomodulatory lead cumamide cucumarioside A2-2, based on the holothurian triterpene glycoside, possesses the ability to inhibit development of tumors, demonstrates significant antitumor activity in vivo, and exhibits synergistic activity when combined with 5-fluorouracil at low dosages. The aim of this study was to investigate the influence of cucumarioside A2-2 upon Ehrlich ascites carcinoma (EAC) tumor cell resistance by analysing the net uptake and intracellular accumulation of fluorescent Calcein AM, as well as the cytostatic drug doxorubicin.

For this purpose, spectrofluorimetry and confocal microscopy techniques were used. Cucumarioside A2-2 was isolated from the Far Eastern holothurian Cucumaria japonica, and kindly provided by Drs. Avilov and Silchenko (PIBOC FEBRAS, Vladivostok).

Cucumarioside A(2-2 influences tumor cell viability at low concentrations. The EC50 established by non specific esterase assay and MTT assay was 2.1 and 2.7 mM, respectively. In the non-cytotoxic range of concentrations, cucumarioside A2-2 blocked the activity of P-glycoprotein of EAC cells, and increased the accumulation of the probe Calcein AM in the cytoplasm. At a concentration of 1 nM, the fluorescence of the probe in the cytoplasm was increased up to 56% compared to the control. Using confocal microscopy, we found that incubation of EAC with cucumarioside A2-2 at concentrations of 1–100 nM for 2 h lead to a marked increase in accumulation and concentration of doxorubicin in the nuclei of tumor cells. In the same range of concentrations, the substance significantly inhibited the efflux of doxorubicin from tumor cells.

We show that cucumarioside A2-2 not only demonstrates significant antitumor activity as a single substance, but in addition has the ability to inhibit drug efflux from tumor cells. This could make it a valuable substance in drug resistance and combinations with other well established cytotoxic drugs.

SW06.S26–12
The activity of cytosolic alanine aminotransferase 2 is regulated by fucose-binding protein through protein–protein interaction
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Up-regulation of cytosolic alanine aminotransferase 2 (cALT2) expression under gluconeogenic conditions leads to reversible transamination of l-alanine and alpha-ketoglutarate to form pyruvate and l-glutamate in the liver of gilthead sea bream (Sparus aurata). To gain insight into the posttranslational regulation of cALT2 activity, we performed a yeast two-hybrid assay to identify proteins that may interact with cALT2. Screening of a S. aurata cDNA library fused to the GAL4 transcriptional activation domain using cALT2 fused to the GAL4 binding domain as a bait, allowed us to isolate a cDNA fragment corresponding to fucose-binding protein (FBP) as a potential cALT2 interacting protein. Rapid amplification of cDNA ends (RACE)-PCR led us to obtain the full-length coding sequence of S. aurata FBP. Cytosolic colocalization and interaction between full-length FBP and cALT2 in SBL cells was confirmed by measuring Fluorescence Resonance Energy Transfer (FRET) efficiency using the acceptor photobleaching technique in a confocal microscope. Transient co-
transfection of SBL cells with cALT2 and FBP expression plasmids revealed that FBP increases the activity of cALT2. In conclusion, since FBPs are known to play major roles in pathogen-recognition in aquatic species, our findings point to an important contribution of cALT2 expression during the immune response to infection.

This work was supported by BIO2009-07589 (MCI, Spain) and AGL2012-33305 (MEC, Spain) grants. MG is recipient of an APIF (Universitat de Barcelona, Spain) fellowship.

**SW06.S26–13**

**Effect of cadmium and transient hypoxia on mitochondrial function and oxidative stress in the Eastern oysters, *Crassostrea virginica***

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Hypoxia and heavy metal contamination are important environmental stressors in estuaries worldwide that can negatively affect performance and survival of estuarine inhabitants. The Eastern oyster, *Crassostrea virginica*, is a keystone species in estuarine communities of the western Atlantic and is commonly exposed to diurnal and seasonal hypoxia and to elevated levels of trace metals including cadmium (Cd). We determined the effects of combined exposure to Cd (30 days at 50 μg/l Cd) and transient hypoxia and the resulting damage to mitochondria and subsequent effects on antioxidant defenses in oysters. Control and Cd-exposed oysters were exposed either to normoxia (21% O2) or to short-term hypoxia (24 h at <1% O2 for oxidative stress) or long-term hypoxia (6 days <1% O2 for mitochondrial functions) followed by a 1 h period of recovery at 21% O2. Respiration, membrane potential(ΔΨ), γ-dependent kinetics of three major mitochondrial subsystems, as well as total antioxidant capacity (TAOC) and concentrations of oxidative stress markers (protein carbonyls, malondialdehyde- (MDA-) and 4-hydroxy-2-nonenal (HNE)- conjugates of proteins) were measured in control and Cd-exposed oysters during normoxia, hypoxia and 1 h of reoxygenation. Preservation of mitochondrial membrane polarization and ATP generation was observed in control individuals following transient hypoxia and reoxygenation. Exposure to Cd abolished the putative adaptive responses of mitochondria to the stress of hypoxia/reoxygenation and increased the TAOC profile. In spite of this increase in TAOC, damage to proteins and lipids was observed. While oysters are well adapted to confront transient hypoxia and reoxygenation, Cd may negatively affect their survival under these conditions. Supported by the National Science Foundation award IOS-0921367.

**SW06.S26–14**

**New immunomodulators from sea cucumbers: molecular mechanisms of action**

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The holothuroid triterpene glycosides at micromolar concentrations exhibit strong cytotoxic activities against different cell types. The basis of the glycoside cytotoxicity is their ability to interact with cellular biomembranes and form nonselective ion-conducting complexes with ω-unsaturated sterols. As result the disrupting of ion homeostasis and osmolarity followed by lysis and cell death take a place. On the other hand, some glycosides demonstrate a pronounced immunostimulatory effect at sub-toxic nanomolar concentrations. Present work demonstrates that incubation of immune cells with the cucumarioside A2-2 from the Far Eastern sea cucumber *Cucumaria japonica* results in their activation. At nanomolar concentrations cucumarioside A2-2 is associated only with high affinity receptors. Using confocal microscopy, flow cytometry, Ca2+-imaging and electrophysiology (patch-clamp) it was found by us that the most likely membrane targets of this glycoside can be purinergic receptors of P2X family (predominantly P2X1 and P2X4). Structural models of A2-2–mP2X1 and -mP2X4 complexes generated by *in silico* modeling disclosed that cucumarioside A2-2 and ATP binding sites are localized at different areas of extracellular receptor domain. Molecular Dynamics simulations results revealed that cucumarioside A2-2 action on the receptor turn those binding site residues connected through β-strands by H-bonds network. These findings and experimental data suggest that cucumarioside A2-2 can act as an allosteric regulator which able to withdraw purine receptor inactivation by extracellular ATP and provide a recovery of Ca2+-conductivity of macrophage membrane. Such type of glycoside interaction with receptors may trigger an activation of Ca2+-signaling pathway that initiates the amplification of expression of certain intracellular target proteins involved in key stages of immune cell physiology. This leads to increasing in cell adhesion, spreading, motility and proliferation, ROS formation, rise in lysosomal activity, phagocytosis, elevated synthesis of some cytokines and pathogenic microorganism killing. Ultimately, an activation of cellular immunity and magnification of the organism resistance to various opportunistic infections is appeared under glycoside action, as it was recently demonstrated by us experimentally.

**SW06.S26–15**

**Regulation of the carbon metabolism by high-light in the marine diatom *Phaeodactylum tricornutum*: a transcriptional approach**

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The current interest in diatoms is manifold as they are key players in geochemical cycles and produces many valuable compounds such as polysaccharides, lipids, polyunsaturated fatty acids, pigments, biofuels … Their particular gene enrichment, inherited from various types of organisms has contributed to the construction of metabolic networks very distinct from those found in other photo-synthetic organisms. Although metabolic pathways that contribute to energy management in diatom cells have been identified, direct responses of the different pathways, their contributive flow and their integration with one another are not enough known, yet central and fundamental for the understanding of diatom ecology but also for the improvement of the use of diatoms as biotechnological tools. To understand these networks, how they are linked, and how deviation impacts the management of carbon atoms, we have compared mRNA expressions by real-time PCR of 70 genes in *Phaeodactylum tricornutum* cultured under several monochromatic light intensities: an optimum intensity (300 μmol/m/s), a low intensity (30 μmol/m/s) and a high intensity (1000 μmol/m/s). The genes studied code for more than 30 key proteins involved in the carbon metabolism in the different cell compartments (mitochondria, chloroplast and peroxisome). Indeed, many parts of the biochemical networks are redundant and numerous enzymes isoforms coexist with different roles.
(for example the enzyme PGAM is coded by seven genes located in three cellular compartments), rendering the analysis even more difficult. Transcriptomic results were integrated on global metabolic schemes to complete our understanding of the cell functioning under light stress.

**SW06.S26–16**

*In vitro anticancer activity of mycalamide A – a metabolite of marine ascidian *Polysiphonia* sp.*  

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Marine flora and fauna is a rich source of natural compounds that possess potent cancer preventive as well as cytotoxic activities. We studied ethanol extract of the ascidian *Polysiphonia* sp., that was selected in a screening process due to its cytotoxic activity against the human cancer HeLa cell line. Cytotoxicity-guided fractionation of the extract resulted, among others, in the isolation of the previously described mycalamide A.  

Mycalamides and related compounds are inhibitors of protein synthesis and show apoptosis-inducing activity. Initially, they were isolated from the marine sponges *Mycale* sp. (mycalamide A, B and D), *Stylitis* sp. (mycalamide C), *Theonella* sp. (onnamides) and *Dicodorina* sp. (theopederins). Previously, mycalamide A was shown to be rather toxic, putting into question its potential as a cancer therapeutic. However, cancer preventive activity of this compound at lower concentrations has so far not been examined. In the work presented here, we investigated the cancer preventive and pro-apoptotic properties of mycalamide A.  

We report isolation of mycalamide A from a representative of the subphylum Tunicata (family Didemnidae) for the first time. This finding strongly supports the hypothesis that symbiotic bacteria are the most likely origin of mycalamides and related compounds in marine invertebrates. In murine B6 C141 P+ cells, mycalamide A was found to induce caspase-3-dependent apoptosis at nanomolar concentrations, at lower concentrations substance exerted mainly antiproliferative activity. Mycalamide A is able to inhibit EGF-induced neoplastic transformation at concentrations almost 50 times lower than the cytotoxic. The compound inhibits transcriptional activity of the oncogenic nuclear factors AP-1 and NF-kb, which at least partially can explain the observed inhibition of EGF-induced neoplastic transformation. Induction of phosphorylation of the kinases MAPK p38, JNK, and ERK was found at high concentrations of mycalamide A. In summary, the drug shows promising potential for both cancer-prevention and cytotoxic therapy and should be further developed.

**SW06.S26–17**

*Kinetics and mechanisms of light stress recovery in the diatom *Phaeodactylum tricornutum*.*  

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More than 70% of Earth is covered by oceans in which diatoms represent the major group of microalgae. Exposure to high-light results in the formation of ROS. To cope with, diatoms trigger the dynamic dissipation of the absorbed energy into heat: the nonphotochemical quenching (qN) of the chlorophyll fluorescence, which mostly rely on the xanthophyll cycle (XC). The XC consists in the reversible deepoxidation of diadinoxanthin (Ddt) to diatoxanthin (Dtt). The deepoxidation requires lumen acidification. A deeper knowledge in the functioning of qN is needed to understand diatom physiology and ecology as qN intensity may constitute a functional trait of the diversity of algae. This contribution deals with qN (not NPQ) analysis and the mechanisms involved in. qN was triggered by illuminating the algae with white light (1000 µmol/s/m) during 7 min. Then, the algae were kept in the dark and the actual qN values were probed using saturating flashes. Using an original method of nonlinear regression analysis of the qN relaxation kinetics, three components were identified in diatoms. The fast (s time-range) and slow (h time-range) components can be fitted with monoeXponential curves whereas the intermediate component (min time-range) could not. The identification of the main mechanisms on which each phase relies was determined using metabolic inhibitors. NH4Cl, a ΔpH building inhibitor, suppressed the intermediate component, demonstrating that it is due to the ΔpH-buildup. To determine if the ΔpH is the only mechanism involved in the intermediate component, algae were treated with DTT, an inhibitor of the XC. In this condition, the magnitude of the intermediate component was recorded, demonstrating the involvement of both mechanisms in qN. Simultaneously, the magnitude of the fast component increased similarly suggesting that the (XC) is mostly involved in. A participation of the ΔpH cannot be completely excluded as the half-time of this component is much longer in the presence of NH4Cl than with DTT. In contrast, DTT but not NH4Cl affects the slow component. In this contribution, we revealed that the three components are linked with the ΔpH and/or the XC activity.

**SW06.S26–18**

*Studies on the bioremediation of oil and oil derivatives using the bacteria on the marmara and the Black seas.*  

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Decontamination of oil and oil derivatives, which cause environmental pollution and effect biological equilibrium dramatically, has also great significance for natural resources and applied microbiology.  

In recent years, it is known that effect of oil derived hydrocarbon pollutants and related to high toxic compounds increase in aquatic and terrestrial ecosystems. Removal or be converted to different forms of oil hydrocarbons via bacteria has become an important issue.  

In this study, the aim was to determine oil and oil derivatives degradation ability of bacteria isolated from our seas (Sea of Marmara and Blacksea) and identification of degradative bacteria to the species level by using classical microbiological and biochemical methods. Bacteria isolated from these seas in the course of our study are our natural resources. These precious resources will be used for bioremediation of toxic compounds at our seas. This will be an important step in evaluation of resources that we have.

**SW06.S26–19**

*Approaches to cell cycle activity study in *Asterias rubens* L.*  

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Echinoderms represent a basal group of non-chordate invertebrate Deuterostomes, characterized by outstanding potential for regeneration. That is why they provide attractive experimental
models to study the fundamental mechanisms of regeneration. One of the main questions that need to be resolved is the characterization of adult animal stem cells that can be involved in repair and regenerative processes. More important properties of these cells are self-renewal potential and multilineage differentiation (Rinkevich, Matranga, 2009). So far arm regeneration of the crinoid Antedon mediterranea represents the most developed echinoderm model, where the problem of competent cells are well established (Candia-Carnevali, 2006).

In crinoids two types of presumable stem cells, involved in regeneration, were identified as amoebocytes and coelomocytes, their proliferative activity was confirmed by BrdU incorporation. Amoebocytes (resident stem cells) are capable to migration along the brachial nerve towards the wound area, where they undergo an extensive local proliferation. Coelomocytes (circulating stem cells), freely moving in the coelomic fluids and produced by the proliferating coelomic epithelium.

Asteroids regenerate at a much slower rate than other echinoderms (Mladenov et al. 1989; Moss et al., 1998). Regeneration of seaweeds proceeds mainly as morphallaxis, i.e. implies the migration of differentiate or undifferentiate cells from depots to injury sites; in this case the cell divisions play a minor role. However, we described in Asterias rubens L. small cells with high nucleo-cytoplasmic ratio able to proliferate in coelomic epithelium (CE) and in subpopulation of cells, faintly bound with CE (CE-F). These cells demonstrated boundary position between CF and CE and capability to migration from CE to CF. The more important characteristic of these cells is the maintaining of proliferative activity in vitro at cultivation during long time (2 months and more) as was shown by BrdU incorporation assay and immunofluorescent analysis after staining of CF, CE and CE-F cell suspensions with phospho-histone H3 antibody, marker of mitotic cells. To further characterize proliferative activity of seaweed cell populations we undertook the comparative analysis of CF, CE and CE-F cell populations by flow cytometry, and immunofluorescent and western-blot analysis of cell population with human antibodies against cell cycle proteins.

We showed the presence of cells in G2-phase of the cell cycle in subpopulation of CE-F cells. The cells stained by anti-Cyclin D and anti-retinoblastoma antibodies were found among CC and CE cells. The antibodies staining was revealed in cytoplasm as well as in nuclei. Western blot analysis confirmed the presence of these cell cycle regulatory proteins in various cell populations. These data support the hypothesis of progenitor properties of small cells with high nuclear-cytoplasmic ratio and demonstrate the perspectives of cell cycle study of small cells with high nuclear-cytoplasmic ratio and demonstrate. These data support the hypothesis of progenitor properties of these cell cycle regulatory proteins in various cell populations.

SW06.S26–20
Exploring the molecular properties of marine mixotrophic protists by a multidimensional scaling
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Mixotrophic organisms that combine autotrophy and heterotrophy are widespread in marine habitats all over the world. Their ecological relevance has been recognized for a long time. However, quantifying the elemental fluxes mediated by mixotrophs still remains a challenge. In many ways this is due to the lack of comprehensive knowledge on the molecular grounds of the metabolic mode. In fact, mixotrophy is a spectrum of various nutritional strategies which can be driven by different molecular machinery and regulated in different ways, but what do they have in common? Unfortunately, it is extremely laborious to answer this question by means of laboratory studies because of the enormous diversity of mixotrophs and time-consuming methods. We used a multidimensional scaling (MDS) of the functional genes that belong to mixotrophs as well as pure autotrophs and heterotrophs in order to reveal some qualitative trends in their distribution among these groups. Thus, MDS can provide us with the information necessary for producing targeted hypotheses on the nature of mixotrophy, that can be tested in the laboratory. Previously, a similar approach has been successfully applied in molecular ecology of bacteria. In the case of protists it is more difficult task, because there is only a low number of completely sequenced protistan genomes, which limits our possibilities and imposes a bias on the results. However, we believe that this obstacle will be overcome in the nearest future. In the present work we show the results of MDS based on currently available data and discuss whether this approach is useful for unraveling the metabolic puzzles.

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SW06.S26–21
Effect of antiparasitic drugs on expression levels of metabolic enzymes and drug resistance proteins in Caligus rogercresseyi
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Sea lice, Caligus rogercresseyi, a copepods ectoparasite, severely affects the salmon farming industry in Southern Chile, reducing the health status of fish and producing both direct and indirect economic losses. Currently, decreased treatment efficiencies or drug resistance have been observed for the drugs used against C. rogercresseyi infections, principally for emamectin benzoate and pyrethroids. Drug resistance in parasites can result from a number of different molecular mechanisms, including changes in drug metabolism and drug distribution in the parasite.

The aim of this study was to determine the presence of metabolic enzymes and MDR proteins, and the effect of antiparasitic treatments on the expressions levels of these enzymes and proteins in total homogenates of the parasites by Western blot, which could be involved in the drug resistance observed. We study the expression of CYP3A, CYP3A27, FMO, GST, Pgp and MRP1 proteins. Up-regulation was observed for the expression for most proteins analyzed after antiparasitic treatments. Nonetheless, CYP3A expression was not detected in this parasite.

These results suggest that Caligus rogercresseyi could regulate the expression of proteins involved in metabolism, distribution and elimination of xenobiotics and thus affect the pharmacokinetics of the antiparasitic, which could be involved in the drug resistance observed in this parasite.

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SW06.S27
Biochemistry of Plants (VI–S27)

SW06.S27–1
Biointeractomics of cytochrome c under programmed cell death in plants and humans
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Cytochrome c (Cc), a small soluble hemoprotein, is highly conserved among evolution. In mammals, Cc plays a dual role in cell
life and death: Under homeostatic conditions, Cc is retained inside the mitochondria and acts as an electron shuttle in the electron transfer respiratory chain. Upon apoptotic stimuli, however, Cc is released into the cytoplasm as to serve as an essential key factor by binding to Apaf-1 and further assembling the apoptosome, the machinery responsible for activation of caspases-3. The mitochondria-to-cytoplasm Cc translocation has been long considered as a random event, although it is an evolutionarily conserved process even in organisms in which the apoptosome assembly is independent of Cc or in which the apoptosome is missing as in plants. These findings, along with the fact that apoptosis remains active in Apaf-1 knockout mutants but not in Cc knockout mutants 2,3, lead one to wonder if cytoplasmic Cc could play other putative signaling functions.

To better understand the role of Cc in the onset of apoptosis and to harmonize the different phenotypes of Apaf-1 and Cc knockout mutants, we have developed a proteomic approach based on affinity chromatography with human or plant Cc as bait. A total of 24 and 10 Cc partners in human and plants cell extracts, respectively, have been identified. Their in vitro interaction with Cc, and cellular localization, were further analyzed by Bimolecular Fluorescence Complementation (BiFC). Finally, the binding affinity constant for some of these complexes has been determined by Surface Plasmon Resonance (SPR).

Altogether, our results open a new ways to understand the Cc-dependent activation and progression of programmed cell death in plant and human cells.

Acknowledgements: This study was funded by the Spanish Ministry of Economy and Competiveness (Grant No. BFU2009-07190/BMC and BFU2012-31670/BMC) and the Regional Government of Andalusia (BIO1918).

References:

SW06.S27–2

Functional genomics and phosphoproteomics of stress responses in cyanobacteria

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Modern methods of examination of gene expression involve the use of DNA microarrays to study gene transcription and mass spectrometry analysis of proteins to study and identify the translation products of the corresponding genes.

The genome of the cyanobacterium Synechocystis sp. PCC 603 carries 47 genes for histidine kinases, and 45 genes for response regulators. Typically, inactivation of genes for sensors and/or transmitters, leads to inability of cells to induce a number of stress response genes. This allows identification of genes that are controlled by such sensor. This strategy was applied to identify sensors of low temperature, salinity and hypersomotic stress, deficit in manganese ions, etc.

Unlike sensor histidine kinase, serine-threonine protein kinases (STPKs) are involved in the regulation of basic physiological processes in prokaryotes (motility, metabolism at high temperatures, etc.). Mutations in genes serine-threonine protein kinases rarely lead to visible changes in the transcription of genes and often expressed in the inability of the phosphorylation of individual proteins. The genome of Synechocystis contains 12 genes encoding STPKs. MALDI-TOF spectrometry identified several proteins as the targets for phosphorylation by the STPKs. Among them, there was a small co-chaperonin GroES. Expressed and purified GroES was not phosphorylated in the mutants defective in three STPKs – SpkC, SpkF, or SpkK. Complementation of any of these mutations restored GroES phosphorylation. These STPKs operate one after the other, creating a cascade of phosphorylation. In wild-type cells and histidine Hik34 and transcription factor HrcA repress transcription of groESL operon at the optimum growth temperature. However, GroES and GroEL were found in cells grown at normal temperature. This indicates that repression of transcription by the Hik34 and/or HrcA is incomplete.

Systematic analysis of mutant libraries of cyanobacteria with DNA microarrays and phosphoproteomics allows identification of sensory systems of cells, as well as other systems of phosphorylation involved in the regulation of cellular processes.

SW06.S27–3

Effect of endemic plants extracts on biological macromolecules

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Reactive oxygen species (ROS) are generated during normal aerobic metabolism, but they may be toxic in higher concentrations causing a great damage of macromolecules such as DNA, proteins and lipids. Oxidative stress which usually generates increased amount of ROS play an important role in an etiology and pathogenesis of various diseases such as cardiovascular disease, cancer, diabetes and neurodegenerative disease. Cells possess mechanisms for radical detoxification that are not full effective in case of drastic stress conditions. Some compounds with important antioxidant activity originated in food and dietary supplements may have protective effect on ROS caused damages. Thus, numerous epidemiological and clinical studies support hypothesis that regular consumption of products reach in antioxidants have protective effect in diseases mediated through deleterious free radical chain reactions. Medicinal plants which have been used in traditional medicine for many years are particularly in focus, although their biological activity and effects on biological macromolecules have not been investigated yet. Herein we investigated potential protecting effects of four endemic plants extracts (Teucrium arardinum L., Melikia petraea (Tratt.) Griseb, Micromeria croatica (Pers.) Schott and Rhamnus intermedia Steud. et Hochst) against ROS damaging of important biological macromolecules: lipids, proteins and DNA. Content of polyphenolic compounds (total phenol, flavonoids, hydroxyccinnamic acids, proanthocyanidins and individual phenolic acids) was determined in plant extracts using spectrophotometer and UPLC-MS/MS. Furthermore, in vitro antioxidant activities of plant methanol extracts were screened using DPPH, ABTS and FRAP methods. All four tested extracts showed high in vitro antioxidant capacity and DNA protection effects. However, their effects on protein and lipid protection were diverse and will be discussed.

SW06.S27–4

Non-cell-autonomous pathway protein binds tobacco mosaic virus movement protein

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Macromolecular cell-to-cell traffic in plants involves plasmodesmata. The mechanism of intercellular transport is highly adjustable and non-cell-autonomous-pathway protein (NCAPP) is reported to be one of its regulators. This 40 kDa protein is crucial for trafficking of specific non-cell-autonomous proteins (NCAPs) such as CmPP16 and the tobacco mosaic virus movement protein (TMV MP) [J.-Y. Lee et al., 2003]. Recently it has been shown that methanol stress signal can induce dramatic raise of Nicotiana benthamiana...
Copper amine oxidase and peroxidase: interacting enzymes in *Euphorbia characias* latex

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*Euphorbia characias* is a spurge belonging to the Euphorbiaceae family. It commonly occurs in various habitats (rocky hillsides, along road verges, in open woods and in olive groves) in vast areas of the Mediterranean basin. Selecting this shrub as a complementary experimental model, we have begun to disclose the properties of several components of the enzymatic machinery present in its latex. Some of these proteins had been purified and characterized.

One of these proteins is an amine oxidase [amine: oxygen oxidoreductase (deaminating; copper containing); EC 1.4.3.6] (*Euphorbia* latex amine oxidase; ELAO) and the other is a catalytic peroxidase (*EC* 1.11.1.7; donor: hydrogen peroxide oxidoreductase). ELP is a calmodulin–binding protein and is activated by the calcium/calmodulin (*Ca*²⁺/CaM) system.

The metabolism of arginine is varied. Arginine is attacked very efficiently by arginase, arginine decarboxylase, NO synthase. Arginase plays a degradative role on arginine yielding urea and ornithine. The last is a good substrate for ELAO yielding glutamate-5-semialdehyde. Arginine decarboxylase produces agmatine from arginine, and again agmatine is a good substrate for ELAO. Moreover arginine and ornithine are precursors for the synthesis of putrescine, spermine and spermidine and these di- and polyamines are really very good substrates for ELAO. Finally, ELAO, acting on arginine and yielding glutamate-5-semialdehyde, ammonia and urea, represent an alternative metabolic pathway of arginine [1].

The cellular metabolism of polyamines catalyzed by ELAO generates dihydroxyethanes and H₂O₂. Scavenging of H₂O₂ is mediated by a complex network of enzymes, including the Ca²⁺/CaM-regulated ELP which can utilize NO as a second substrate generating H₂O₂, NO⁻ and NO₂⁻. Polymamines, derived from arginine by arginase and arginine decarboxylase, can increase the NO amount. A variety of biotic and abiotic stresses may elicit the transient increase of NO by both nitrite or arginine pathways, the last catalyzed by nitric oxide synthase. Moreover, both NO and H₂O₂ can regulate calcium homeostasis by activation of plasma membrane Ca²⁺-permeable channels. Calcium ions, normally necessary for expression of the full activity of the ELP, inhibit the enzyme activity in the presence of NO or of the pseudohalide thiocyanate (SCN⁻) as second substrates. When an excess of the SCN⁻ and H₂O₂ was added to ELP in the presence of 10 mM Ca²⁺, free radicals derived by the oxidation of SCN⁻ form dithiocyanogen (SCN₂), (due to two successive one-electron transfer from SCN⁻ and (SCN₂) is hydrolyzed to yield CN⁻ [2]. This latter species acts as a reversible inhibitor of ELP and NO switches off the CN⁻ inhibitory effect by a singular mechanism involving the production of ammonia and CO₂ [3]. Finally, NO is an irreversible inhibitor of ELAO.

References


Comparative analysis of different parts of *Vicia faba* for production of a protein isolate with high antioxidant activity, L-DOPA and phenolics content

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Faba bean (*Vicia faba*) is an important member of the legume family with many useful features. It is a rich source of protein, complex carbohydrates, dietary fiber, choline, lecithin, minerals and phenolics. These ingredients have important use in diseases such as hypertension, renal failure and liver cirrhosis. Fava beans are especially rich in levo-dihydroxyphenylalanine (L-DOPA), the precursor of dopamine which is being used in the management of Parkinson’s disease. Parkinson’s disease is a progressive neurodegenerative disease caused by insufficiency of dopamine in the
Transcription factors (TFs) are regulatory proteins that have played a pivotal role in the evolution of eukaryotes and are believed to display great biotechnological potential. We performed the functional characterization of transcription factors following strategies designed to examine the biotechnological potential of selected TFs in crop species. A collection of transgenic Arabidopsis lines each of which express a single TF under the control of an inducible promoter were phenotypically characterized in relation to a large number of developmental and stress-related traits. Abscisic acid (ABA) plays a major role in regulating physiological processes in the plant such as seed development and germination, and mediating the response of vegetative tissues to osmotic stress. Therefore, ABA regulated TFs were selected for further characterization. Determination of the DNA binding specificity of the ABA regulated subset of TFs following a microarray-based approach provided information on TF binding specificity, this, together with bioinformatics analysis of the transcriptome data, help associating TFs to target genes relevant to a given biological process. The assessment of the biotechnological importance of ABA involved TFs identified because of regulation of agronomical/economical relevant traits, will be carried out by testing the effects of controlled overexpression or down-regulation of the ortholog TFs in crop species.

**SW06.S27–9**

**Copper induced physiological, biochemical and bimolecular responses in B73 maize**

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The effects of different copper (Cu) concentration for 24, 48 and 72 h of treatment were studied in roots and leaves of B73 maize in hydroponic condition.

A decrease of root elongation was observed increasing Cu concentration. On the contrary, the lignification increased with the rise of Cu, showing the maximum value in presence of Cu 2 mM after 24 h and 48 h treatment, and Cu 5 mM after 72 h treatment.

The superoxide dismutase, peroxidase and catalase activities increased with increasing Cu concentration. Peroxidase and catalase activity displayed higher values after 72 h of Cu treatment in the roots, while there was a reduction of these activities in the leaves.

In roots, a change in isoenzyme profile of SOD and POD leads us to consider this variation as plant’s response to Cu stress condition.

Lipid peroxidation was found to increase in Cu excess that induced oxidative stress, showed the most damage in presence of 5 mM of Cu after 72 h of treatment both in leaves and roots.

Protein, antioxidant and phenol content showed small differences in the roots and a clear reduction in the leaves after 72 h treatment.

Finally a down-regulation of genes PAL, HCT, 4CLb, CCCaOMT, F5H, CCCRb and Lac III only in Cu absence and the up-regulation of Lac III in presence of 5 mM Cu confirms the reduction of lignin without Cu and greater lignification in presence of Cu 5 mM in compared to treatments in presence of Cu 1 mM.

These results indicated that Cu deficiency and excess induced changes in B73 metabolism in which the antioxidant enzymes could be important biomarkers as well as a defence against Cu induced stress.

Furthermore, this knowledge can help to get more information on Cu tolerance in B73 that is a model maize due to its completely sequenced genome.

**SW06.S27–10**

**The new plant protein involved in cell death**

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Although the yield of natural and recombinant proteins could reach high levels in plant cells, the mechanism by which tolerance to overproduced proteins is achieved remains unclear. We previously showed on transient expression system that rapid protein synthesis can result in leaf necrosis with the appearance of mitochondria and dead cells. To understand the mechanisms by which protein overproduction can lead to cell death, we selected and isolated a gene encoding protein with 50% homology to Kunitz trypsin inhibitor and later named it plant death factor (PDF). We showed that PDF mRNA accumulation could be induced by GFP mRNA produced both from viral and non-viral vectors. Biologically, PDF mRNA increase is likely to accompany cell

brain. L-DOPA administration is a standard mode of therapy for Parkinson’s disease. Fava bean consumption has been shown to increase the levels of L-DOPA in serum. It also improves the motor performance of the patients without any side effects. Since synthetic L-DOPA has some side effects, a natural L-DOPA source is more desirable. In a recent clinical case, it was shown that fava bean consumption prolonged the ‘ON’ periods in patients who have ‘ON-OFF’ fluctuations. These prolonged ‘ON’ periods were not observed when synthetic L-DOPA was administered at high doses. There might be other ingredients in the fava bean. Phenolics are the second important metabolites interacting with the environment. Fava beans contain highest amount of phenolics in the legume family. Plant phenolics have potential health benefits due to their antioxidant properties such as electrophilic scavenging and inhibition of reactive oxygen species (ROS). Biosynthesis of L-DOPA starts with the amino acid tyrosine. Tyrosine is a product of pentose phosphate pathway. This oxidative pathway provides the carbon skeleton for shikimate pathway that leads to the phenylpropanoid pathway with the entry of phenylalanine. Chorismate is the end product of the shikimate pathway and that supplies the branch point for synthesis of the three aromatic aminoacids (Phe, Tyr, Trp). All phenylpropanoids are derived from phenylalanine that also supplies the branching point for synthesis of L-DOPA via tyrosine. In our study, seeds of fava bean (Vicia faba) were germinated by utilizing various processes. Seed sprouts were then separated into their parts (leaf, stem and cotyledon). Dry seeds were homogenized before assessment. The homogenates were assayed for their total phenolic content, antioxidant activity (ABTS and ORAC methods), superoxide dismutase (SOD) activity, proline levels, glucose 6-phosphate dehydrogenase (G6PDH) and guaiacol peroxidase (GPX) using spectrophotometric methods. L-DOPA was quantified using HPLC. Our preliminary data have shown that Vicia faba protein isolate with high L-DOPA, total phenolic content and enhanced antioxidant activity could be used as a source of high quality functional and nutritional components. Thus, its low cost and easy availability would make a good option for dietary support in Parkinson’s disease.
death and reduce mRNA and/or protein accumulation. To identify the correct outcome, we created PDF-encoding genetic constructs and obtained E. coli producing PDF. We showed that recombinant PDF protein had no trypsin inhibiting activity. Agrinjection experiments with PDF sequence in antisense polarity (asPDF) showed that host PDF gene knockdown resulted in an increase of viral vector-directed protein production. We believe that PDF constrains excessive mRNA and protein synthesis and its knockdown leads to the increased protein accumulation.

The synthesis of PDF mRNA may reflect the level of biosynthetic activity of the plant tissue. To obtain experimental confirmation of this hypothesis, we found that in contrast to the leaves, the root system of the plant, and, mainly, root border cells have an increased level of PDF mRNA synthesis. Moreover, it appeared that the amount of PDF mRNA in root tissues of flowering tobacco plants was up to 400-fold higher than in roots of young seedlings. We concluded that the level of PDF gene expression in root cells reflects and characterizes the age of the plant.

**SW06.S27–11**

**Anti-oxidant capacity of Aesculus hippocastanum fruit parts as an anti-aging agent**

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Aesculus hippocastanum may be stated as an important medicinal plant with various bioactivities. The whole seed extract of A. hippocastanum has been shown to be a potent anti-aging ingredient. Many reports suggest a link between skin ageing and active oxygen species, especially when combined with ultra-violet radiation. In this comparative study, methanol extracts of the fruit parts (capsule, seed testa and seed cotyledon) of A. hippocastanum were evaluated for their antioxidant capacity using DPPH and ABTS methods. The seed testa was found as most effective radical scavenger with an EC50 value of 0.016 mg/ml and with a TEAC value of 39,301 µmol/g. Amount of phenolic compounds were searched in order to correlate anti-oxidant activities of the extracts. The seed testa has also revealed the highest amount of 0.644 mg gallic acid equivalence (GAE) in 1 mg of extract. The content of flavonoid expressed as rutin equivalents, varied from 0.027 to 0.482 mg rutin equivalent/g extract. The seed cotyledon showed the highest amount of flavonoid contents followed by the capsule and the seed testa. As oxygen species are associated with cellular damage and inflammation, the use of products containing seed testa, instead of products containing whole seed, may hold greater potential to alleviate damage to the skin. Further studies on the molecular mechanisms of these extracts on preventing UV-induced cellular damage should be carried out in the near future.

**SW06.S27–12**

**Redox regulation of Arabidopsis MEDIATOR: the missing link between transcription factors and their targets**

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In eukaryotes, the mediator multi protein complex relay regulatory information required by RNA polymerase II from DNA-bound transcription factors for the transcription of target genes. This mediator is composed of three core modules and a separable kinase module. Although the function of individual MED subunits has been established in plants, only few comprehensive reports on the functional and transcriptional regulation of MED subunits in response to environmental/abiotic stress have been released. Several processes like responses to light quantity and quality as well as development are unique to plants; therefore it is not surprising that several plant specific transcription factors as well as MED subunits have emerged. Moreover, being sessile organisms, plants are unique in interaction with their surrounding environment both biotic and abiotic which cause them to be constantly subjected to changes in their environment, causing them to alter their metabolism in order to maintain a steady-state balance between energy generation and consumption. Therefore, a tight control is needed to balance these activities and maintain coordination, including reversible redox regulation of proteins by dithiol-disulfide exchange, activation of signaling pathways by ROS-responsive regulatory genes and buffering of ROS by ROS-scavenging enzymes and antioxidant molecules. In this study we demonstrate the involvement of representative MED subunits in redox-dependent regulation of DNA-binding activity of GeBP-like transcription factor. GeBP-like transcription factor was isolated primarily as an associated protein with the mediator complex and was subsequently isolated from control and HL-treated Arabidopsis plants using DNA-affinity trapping approach suggesting its involvement in redox regulation. We show that GeBP-like as well as the med subunits investigated are responsive to reducing and oxidizing agents. We further characterized the domain responsible for DNA binding of GeBP-like protein. Comparative survey on the presence and conservation of Cys residues among different organisms including homosapiens, saccharomyces cervisiae, rice and Arabidopsis revealed conservation of Cys residues among almost all MED subunits within the plant species. Taken together, our data suggest a novel redox conserved mechanism of the mediator complex in plants.

**SW06.S27–13**

**Oxidative processes and defence system functioning in barley seedlings under drought and rewatering**

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Soil drought is one of the main factors limiting plant productivity. Plant responds to water deficiency by overproduction of reactive oxygen species (ROS). Destruction of ROS in plant cells is realized by means of antioxidative system including low-molecular weight antioxidants (ascorbate, glutathione, tocopherols) and antioxidant enzymes. The dynamic of oxidative processes and defense system under prolonged soil drought were investigated in green barley (Hordeum vulgare L.) seedlings. ROS overproduction was registered in green barley leaves cultivated under soil drought since germination which paralleled increase in MDA-products content. The increase in electrolyte output under prolonged drought was observed, suggesting the disturbance of membrane barrier properties. ROS accumulation and lipid peroxidation were accompanied by growth inhibition and photosynthetic pigments destruction. Barley defense system activation under prolonged soil drought was also showed. This was evident in the increase of α- and γ-tocopherol and total glutathione content, in the reduction of ascorbic acid and in the activation of main antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase. Gene expression analysis allowed us to reveal a set of genes encoding defense proteins, antioxidant and subsidiary enzymes overexpressing under
water-limiting conditions. The correlation was stated between drought intensity and osmolytes (such as proline and dehydrins) supply. During rewatering after prolonged drought intensification of oxidative processes occurred. The absence of the elevation in lipid peroxidation products content and plasma membrane permeability suggested that defense system works efficiently. The decrease in reduced ascorbate amount as well as total glutathione content and glutathione reduced to oxidized form ratio enhancement after rewatering confirmed this fact.


SW06.S27–15
2D-PAGE Gel-based comparison of proteomes of two pine species under environmental stress conditions
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Pinus brutia, which is an economically important forest tree species for Turkey, is a drought resistant pine that withstands more aridity and poor soils than most other timber species growing in the same climatic conditions. Pinus sylvestris is another important pine species of Turkey, which resists harsh winter conditions unlike P. brutia. In this study, we aimed to compare the proteomes of those species under drought and cold stresses. Twenty healthy trees from each species were labeled as samples at the same region and elevation. Sampling was performed three times in a year: winter time for cold stress, hot and dry summer time for drought stress and humid and mild spring time as a reference for other two samplings. Humidity and temperature measurements were done for the same region. Carotenoïd and chlorophyll contents were measured for each sample used as indicators for stress conditions. Chlorophyll a, chlorophyll b and chlorophyll (a+b) values were peaked at summer time for P. sylvestris; but, they reached their highest values at spring time for P. brutia. Unlike P. sylvestris, P. brutia showed 40% increase in total carotenoïd content in spring season. With respect to other two seasons, at winter time, proline contents of P. sylvestris and P. brutia were higher at percentages of 58% and 51%, respectively. MDA measurements didn’t show considerable change for both species throughout the sampling time, but overall, they were approximately two times higher for P. brutia. All biochemical and meteorological data supported the presence of environmental stress in summer and winter times. After protein extraction of each sample by using a novel ‘combined’ method for conifer species which was adapted by authors, 2D protein gel electrophoresis was performed. At pH range of 5–8 (provided by IPG strips), 350–500 protein spots were detected on each gel having variable changes as they compared for seasons.

SW06.S27–16
Identification of phenolic constituents from Turkish A. hippocastanum by RP-HPLC
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HPLC isolation and spectroscopic characterization studies were performed for identifying the major constituents of the bark extract of Aesculus hippocastanum L. collected from Turkey. Isolation process was carried out using a semi-preparative RP-HPLC with diode array detector. The major phenolic components of the bark extract were identified by means of analytic RP-HPLC. Isolated compounds were characterized when necessary, along with comparison of available spectroscopic database, by the help of UV-vis, 1H, 13C NMR, FTIR and HR-MS techniques. The isolated compounds A and B, in accordance with the RP-HPLC elution profile, were identified as 6-(beta-D-glucopyranosyl)-(2H)-7-hydroxy-2H-1-benzopyran-2-one and 6,7-dihydroxycoumarin, while the third isolate compound C was characterized as 7,8-dihydroxy-6-methoxycoumarin. Compounds A and B
exhibited very important spectroscopic features with the emission maxima of 515 nm and 445 nm, respectively.

**SW06.S27–17**

Structure and function of bicovalent flavoenzymes from *Arabidopsis thaliana*

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Berberine bridge enzyme (BBE) from California poppy is a paradigm for bicovalent flavoenzymes, where the flavin is linked via a cysteine and a histidine residue to positions 6 and 8 of the isoalloxazine ring, respectively. It catalyzes the oxidative ring closure reaction of (S)-reticuline leading to the formation of (S)-scoulerine as an important intermediate in benzylisoquinoline alkaloid biosynthesis. In BBE, the bicovalent flavin attachment influences the redox potential, protein stability and the positioning of the cofactor in an open and easily accessible active site, thus enabling efficient catalysis.

In order to evaluate the occurrence and distribution of BBE homologs, we performed sequence alignments with a number of plant genomes. This led to the identification of BBE-homologs in various plants, most of which are not reported to produce alkaloids. Some of the genes encoding for BBE-homologs are upregulated during immune and stress response in several species. This led to the identification of BBE-homologs from the model plant *Arabidopsis thaliana* using the YASARA modeling tool with special regard to the active site composition and to residues required for bicovalent cofactor tethering. Furthermore, the BBE-homologs were analyzed on a phylogenetic level and according to their expression levels under different conditions.

Thus, by performing a biochemical and structural analysis of these BBE-homologs we aim to determine the physiological role of these enzymes in planta.

**SW06.S27–18**

Preparation and purification of native geranylgeraniol-18-hydroxylase from *Croton stellatipilosus* Ohba

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Geranylgeraniol-18-hydroxylase is an enzyme that catalyzes the hydroxylation reaction of geranylgeraniol (GGOH) to plaunotol, an antilucretic constituent accumulated in leaf of *Croton stellatipilosus* Ohba. The activity of GGOH-18-hydroxylase was previously reported to be present in the microsomal fraction prepared from leaf [1]. The enzyme activity appeared to be detectable for only a few days causing difficulty in the study of the enzyme properties. Therefore, in this study, we investigated the condition for maintaining enzyme activity in the leaf protein extract for 1 week. This was accomplished by using of the ingredient buffer system for resuspended pellet of 100 mM MOPS pH 7.8, 0.2 M Sucrose, 20%v/v Glycerol, 1 mM DTT, 5 mM L-mercaptoethanol, 0.5 mM EDTA and 0.1%v/v Triton x100, followed by 20 000 g centrifugation to obtain microsomal fraction which contained GGOH-18-hydroxylase activity. The formation of plaunotol using a TLC silica gel separation system of chloroform:n-propanol:ethylacetate:acetic acid in ratio of 59.9:1:40:0.1. The enzyme was purified by heat inactivation and ion exchange chromatography. The obtained results showed that the heating of microsomal fraction at 45°C for 10 min let to the accumulation of an expected monomeric molecular weight protein of 53.4 kDa in 20 000 g pellet fraction as observed by SDS-PAGE. In term of purification, the optimum pH for the binding of the expected protein to QAE sephadex A50 column was found to be 8.5. This pH condition will be applied for a large scale preparation in the future. It is expected that the purified enzyme from these techniques still contain GGOH-18-hydroxylase activity. Presently, the improvement of the technique to monitor GGOH-18-hydroxylase activity and characterizing the NADPH-dependent enzyme is still in progress.

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**Reference**


**SW06.S27–19**

Structure-function study on maize enzymes connected to cytokinin metabolism – nucleoside N-ribohydrolases and aldehyde dehydrogenases

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Cytokinins are the plant hormones and consist of an adenine/adenosine moiety carrying an N6-isoprenoid or aromatic side chain. Their irreversible inactivation by cytokinin oxidase/dehydrogenase (CKO) leads to adenosine/adenine and corresponding aldehyde. Up to now, the fate reaction products remains unclear. This work focuses on two enzyme groups possibly involved in their metabolism – nucleoside N-ribohydrolases (NRHs) catalyzing conversion of adenosine to adenine and aldehyde dehydrogenases (ALDHs) oxidizing aldehydes to corresponding acids.

NRHs are glycosidases that catalyze the excision of the N-glycosidic bond in nucleosides to allow recycling of the nitrogenous bases and ribose. NRHs belong to metalloproteins. We analyzed several NRHs from two model plants – moss (*Physcomitrella patens*) and maize (*Zea mays*) and solved crystal structures of both representatives. The enzyme comprises four Asp residues in a conserved sequence motif DXXDXXXDD at the N terminus involved in catalysis and coordination of a calcium ion at the active site. The binding of ribose moiety is highly conserved but the residues interacting with nucleobase highly vary. Therefore all NRHs characterized so far impose a strict specificity for the ribose moiety, but they exhibit variability in their preferences for the nucleobase (pyrimidine/purine). The substrate specificity is determined by flexible loops over the active site. The interacting residues positioned on a major loop were identified by site-directed mutagenesis. ALDHs comprise a protein superfamily of NAD(P)⁺-dependent enzymes and eliminate biogenic and xeno-
Biotic aldehydes to the corresponding carboxylic acids. We analyzed several ALDHs from maize belonging to families 2, 3, 7, and 10. As a result, we identified enzymes from family 2, which are able to oxidize both aliphatic and aromatic aldehydes produced during cytokinin degradation.

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**SW06.S27–20**

**Evolutionary dynamics of disordered proteins in plants**

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Intrinsically disordered proteins encoded by plant nuclear genomes follow the same general trend of multi-cellular eukaryotes; however, chloroplast-encoded proteins conserve the patterns of Archaea and Bacteria, in agreement with their phylogenetic origin. Our recent investigations suggest that the evolutionary dynamics of the plant nucleus adds disordered segments to genes alike, regardless of their origin, with the notable exception of proteins currently encoded in both genomes [1]. The results also show that the disordered gain is a stochastic phenomenon. In the present work we analyze the distribution of intrinsically disordered proteins along the chromosomes of several representative monocots and eudicots plants. The index of dispersion calculated for the five chromosomes (VMR > 1), a discrete probability distribution. The observed correlations between the frequency of disordered segments (L > 30aa) and the recombination rate measured within different chromosomic regions [2–4], and between the frequency of disordered segments and guanine plus cytosine content (C+G) are shown. The results suggest that higher recombination rates as well as higher number of breakpoints in chromosomal regions can induce higher content of disordered segments in proteins.

**References**


**SW06.S27–21**

**PYL8/PCAR3 is a positive regulator of abscisic acid signaling**

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The phytohormone abscisic acid (ABA) regulates physiologically important developmental processes and stress responses. Recent identification of soluble ABA receptor genes, pyrabactin resistance gene (PYR) and its homologs (PYLs)/regulatory components of ABA receptors (RCARs), have provided a major breakthrough in understanding the signaling mechanisms of ABA. There are 14 PYR and PYL/RCAR genes in Arabidopsis. One of those ABA receptor genes, *PYL8/RCAR3*, is specifically expressed in developing radicles. Constitutive overexpression of it confers ABA hypersensitivity in seed germination. 35S:*PYL8/RCAR3* transgenic Arabidopsis plants also showed strong inhibition of early root growth as compared with the wild type. On the contrary, constitutive RNA interference of *PYL8/RCAR3* showed a resistance of germination and root growth in ABA media. Furthermore, the stomata of 35S:*PYL8/RCAR3* plants had smaller guard cells and apertures than those of wild type plants by approximately 20%, but RNAi plants of *PYL8/RCAR3* had bigger guard cells and apertures than those of wild type plants by approximately 20%. Both overexpression and RNAi transgenic plants confirm that *PYL8/RCAR3* positively regulates ABA signaling during germination and growth.

**SW06.S27–22**

**Quantification of plaunotol and identification of transcript level of genes involved in plaunotol biosynthesis**

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Plaunotol (18-hydroxy geranylgeraniol) is the natural compound from the leaf of *Croton stellatopholis* Ohba. It has been used for the treatment of peptic ulcer. To determine the content of plaunotol present in various stages of the leaf, the technique of Thin Layer Chromatography (TLC) was used. The mobile phase was optimized to obtain a clear separation and plaunotol from other constituents in the crude extracts using chloroform: n-propanol: ethyl acetate: acetic acid (55:9:4:0:1). The highest content of plaunotol was found in the 4th leaf with the amount of 289.60 ± 14.116 μg/leaf, whereas small amounts were detected in shoot and the 1st leaf. These suggested that the fully grown leaf is the main site for plaunotol accumulation. Transcript levels of genes involved in plaunotol biosynthesis such as geranylgeraniol diphasphate phosphatase (GGPP phosphatase) and geranylgeraniol 18-hydroxylase (GGOH-18-hydroxylase) were increased along the leaf developmental stages, suggesting that there is a correlation between the gene expression and plaunotol biosynthesis. The GGOH-18-hydroxylase was cloned and expressed in *E. coli* (BL21 DE3). The expressed protein was approximately 50 kDa and was found in the pellet fraction which indicated that G18H is the membrane associated protein.

**SW06.S27–23**

**The high carbonic acids content variation of macrophytes: *Myriophyllum spicatum* L. and *Elodea canadensis* Michx. from Angara River under hyperthermia and cadmium chloride influence**

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The fatty acid content in studied species has changed insignificantly under hyperthermia conditions (30°C). In *E. canadensis* only the contents of palmitic (C16:0) and palmitoleic (C16:1) acids decreased significant over the 48 h of hyperthermia. In *M. spicatum* only the palmitoleic (C16:1) acid content has decreased. The heneicosanoic (C21:0) acid content has decreased over the 48 h of hyperthermia. In *M. spicatum* only the palmitoleic (C16:1) acid content has decreased. The heneicosanoic (C21:0) acid content has decreased over the 48 h of hyperthermia. In *E. canadensis* from 20.1 to 16.95% and in *E. canadensis* from 24.39 to 23.17%.

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**SW06.S27–24**

**Pyrophosphate-dependent cation ATPase in cyanobacterial photosynthetic membranes**

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The pyrophosphate-dependent cation ATPase was purified from the cyanobacterium *Synechocystis sp.* 6803. The enzyme was solubilized with Triton X-100 and 2-mercaptoethanol and the activity was observed in the range of pyrophosphate concentrations from 1 to 10 mM. The optimal pH for enzyme activity was 8.0. The kinetic parameters were determined in the presence of pyrophosphate as an activator and ATP as a substrate. The enzyme was activated by pyrophosphate with an inhibition constant KI = 0.12 mM. The optimal concentration of ATP was 10 mM. The enzyme activity was inhibited by ouabain and by ouabain congeners with Ki = 0.03 mM. The pyrophosphate-dependent cation ATPase from *Synechocystis sp.* 6803 was highly sensitive to ouabain and its congeners and to hexaammineruthenium(III) chloride, which is a specific inhibitor of Na+/K+ ATPase. The enzyme from *Synechocystis sp.* 6803 was present in the photosynthetic membranes and was activated by pyrophosphate with KI = 0.12 mM.
The treatment of studied species with the solution of cadmium chloride (100 mg/l) for 24 and 48 h has resulted in a change of the fatty acids composition in the tissues of the aquatic plants. The content of fatty acids in M. spicatum has remained close to the control under the action of cadmium chloride, a statistically significant increase has been detected through 24 h exposure with toxicant for myristic (C14:0) and pentadecanoic (C15:0) acids only. The relative content of fatty acids in E. canadensis has changed otherwise. The contents of pentadecanoic (C15:0), heptadecanoic (C17:0), stearic (C18:0), and arachidic (C20:0) acids have significantly increased over 24 and 48 h of the experiment. The content of palmitic (C16:0) and behenic (C22:0) acids have significantly increased by 48 h exposure with cadmium chloride.

In general, the total content of the saturated fatty acid has significantly increased by 48 h of the experiment. The treatment of E. canadensis with a solution of cadmium chloride for 48 h has resulted in a statistically significant increase of the content of the palmitoleic acid isomers sum (C16:1) and the amount of the cis-vaccenic (C18:1n-7) and the oleic (C18:1n-9) acids (Table 2). The content of γ-linolenic acid (C18:3n-3) has significantly decreased over 48 h of the experiment. Changes in mass fractions in E. canadensis fatty acids has led to decrease of the double bond index, however this change has acquired the statistically significant character over 48 h of the cadmium chloride exposure only.

**SW06.S27–24** Cytotoxic effects of *Aloe vera* (L.) Burm. fil. leaf extracts on B16F10 murine melanoma and NIH3T3 mouse embryogenic fibroblast cells

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Referred to as a miraculous plant, *Aloe vera* (L.) Burm. fil. (=*Aloe barbadensis* Miller), member of Aloaceae family, possesses many pharmacological activities. Among the various known therapeutic effects of *A. vera*, recent studies have shown that preparations of the plant leaves have the ability to prevent the growth or to regress certain tumours. The multiplicity of the biological activities of *A. vera* has been attributed to the variety of its chemical components including anthraquinones, glycoproteins, polysaccharides, vitamins and enzymes.

Chemotherapeutic drugs of plant origin are promising strategy for cancer therapy because they might be generally harmless or less toxic than synthetic chemotherapeutic agents on normal cells. The objective of the study was to establish the in vitro effect of *A. vera* leaf extracts on B16F10 murine melanoma and NIH3T3 mouse embryogenic fibroblast cells to ascertain the potential use of these extracts and validate the traditional use.

Water extracts were prepared separately from the leaf skin and gel parts of the fresh plant leaves and lyophilized. Cytotoxicity experiments were done using B16F10 and NIH 3T3 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *A. vera* leaf gel and skin extracts were tested at different concentrations of (1–0.001 mg/ml). The cytotoxic concentrations of extracts that provides 50% inhibition of cell growth (IC50) were calculated from dose-response curve. The cytotoxic effect of *A. vera* extracts were evaluated by comparing the IC50 values of all of cell lines.

Among the two extracts studied, it was shown that *A. vera* gel extract has selective cytotoxic effect on B16F10 cells at 10 μg/ml while it has no effect on NIH3T3 cells in the same concentrations with an even higher IC50 level such as 100 μg/ml. *A. vera* gel is widely used commercially as an ingredient in various cosmetic preparations as well as in a range of healthcare products. The burn and wound healing effect of the gel is very well documented, that is why it is used particularly in skin preparations. Consequently, the high cytotoxic effect of the leaf gel observed in this study in a malign skin tumor type could have an important impact for future research.

**SW06.S27–25** Phytaspases: plant cell death-related proteases with caspase specificity

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Animals and plants employ programmed cell death (PCD) as a mechanism to eliminate redundant and damaged cells. In animals, accomplishment of apoptosis, the most well-understood form of PCD, relies on activation of caspases, a set of highly specific cysteine-dependent proteases with aspartate cleavage specificity. Plant genomes lack caspase orthologous genes. Instead, we recently discovered plant serine-dependent proteases named phytaspases (for plant aspartate-specific proteases). Phytaspases, which are structurally very different from caspaspes, are both involved in PCD and possess characteristic caspase-like cleavage specificity.

Importantly, a strategy employed by plant cells to control phytaspase activity is drastically distinct from the one utilized by animal cells towards caspasps. Whereas caspasps are controlled at the level of proenzyme processing, in the case of phytaspases proenzyme processing/activation occurs autocatalytically and constitutively. To avoid unintended cell death, plant cells exclude (secrete) the mature phytaspase into the apoplast and allow the proteolytic enzyme to re-enter cells upon the induction of PCD. We will discuss biochemical, genetic and physiological evidence for phytaspases from a number of plant species.

**SW06.S27–26** OsNAC5 overexpression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield

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Drought conditions are among the most serious challenges to crop production worldwide. Here, we report the results of field evaluations of transgenic rice plants overexpressing OsNAC5, under the control of either the root-specific (RCc3) or constitutive (GOS2) promoters. Field evaluations over three growing seasons revealed that the grain yield of the RCc3:OsNAC5 and GOS2:OsNAC5 plants were increased by 9–23% and 9–26% under normal conditions, respectively. Under drought conditions however, RCc3:OsNAC5 plants showed a significantly higher grain yield of 22–63%, whilst the GOS2:OsNAC5 plants showed a reduced or similar yield to the non-transgenic (NT) controls. Both the RCc3:OsNAC5 and GOS2:OsNAC5 plants were found to have larger roots due to an enlarged stele and aerenchyma at flowering stage. Cell numbers per cortex layer and stele of developing roots were higher in both transgenic plants than NT controls, contributing to the increase in root diameter. The root diameter was enlarged to a greater extent in the RCc3:OsNAC5, suggesting the importance of this phenotype for enhanced drought tolerance. Microarray experiments identified 25 up-regulated genes by more than 3-fold (p < 0.01) in the roots of both transgenic lines. Also identified were 19 and 18 up-regulated genes that are specific to the RCc3:OsNAC5 and GOS2:OsNAC5 roots, respectively. Of the genes specifically up-regulated in the
RCe3:OsNAC5 roots, GLP, PDH, MERIS and O-methyltransferase were implicated in root growth and development. Our present findings demonstrate that the root-specific overexpression of OsNAC5 enlarges roots significantly and thereby enhances drought tolerance and grain yield under field conditions.

**SW06.S27–27**
The role of mitochondria during realizing of cell death process, induced by subzero temperature, in suspension cell culture of *Saccharum officinarum* L.

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Investigation of features of genetically controlled and well-ordered process of cells destruction, called programmed cell death (PCD), in plants is one of the fastest growing areas of research in recent years. The aim of this study was a possibility of cell death initiation in suspension culture of sugarcane by subzero temperature (−8°C), and detection of changes in the functional activity of mitochondria in the initial stage of activation of cell death process.

This study has been carried out using 8-d old suspension culture of sugarcane (*Saccharum officinarum* L.) during the early exponential phase. The cell culture has been subjected to subzero temperature (−8°C) for 1, 2, 3, and 6 h. It has been shown that the treatment of suspension culture of sugarcane with subzero temperature for 2 h has resulted in a prolonged process of cell death in the culture, which has been observed during 16 h after exposure of sugarcane cell culture to the subzero temperature. At that period of time more than 80% cells died in suspension culture.

Further investigation of the cell death process in suspension culture of sugarcane caused by this type of treatment (−8°C, 2 h) has showed that the induction of the process has been accompanied by increasing content of reactive oxygen species (ROS) in cells and a hyperpolarization of the inner mitochondrial membrane (ΔΨm) has been gradually decreasing for the first 4 h after exposure of cell culture to the subzero temperature. At the same time, significant decrease of intensity of sugarcane cell respiration has been observed. Perhaps, decreases of cell respiration and ΔΨm have been associated with the release of cytochrome c from mitochondria, occurring at the same time. It should be noted that considerable decrease of ROS content in cells during this period of time has not been observed. Thus, it could be concluded that the subzero temperature induces the prolonged process of cell death in suspension culture of sugarcane, which might be caused by oxidative stress and realizes with the participation of mitochondria.

**SW06.S27–28**
Changes in the activity of alternative pathway of respiration, caused by overexpression or reduced expression of the aox1a, affect to the frost resistance of Arabidopsis plants

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Alternative oxidase (AOX) is a terminal oxidase of the plant respiration chain and catalyzes ubiquinol oxidation, leading to reduction of O2 to H2O. AOX is not a proton-pumping enzyme, and transport of electrons through the AOX bypasses proton-pumping complexes III and IV respiratory chain. The role of AOX in response of plants to cold has been shown but frost resistance has not been studied. Therefore we have explored the frost resistance (−2, −4, −6, −8°C, 2 h) and physiological and biochemical parameters of control and hardened plants of Arabidopsis, using the overexpression (sense) and anti-sense Aox1a mutant lines. Plants of *Arabidopsis thaliana* (L.) Heynh of lines Col-0 (WT plants, ecotype Columbia), XX-2 (sense line, NASC N6591) and AS-12 (anti-sense line, NASC N6707) have been used. Seeds of mutant lines have generously been given by V.I. Tarasenko.

It has been shown that differences in the AOX activity did not influence on the carbohydrates, proline, reactive oxygen species and anthocyanins contents, but affected to activities of antioxidant enzymes (total superoxide dismutase – SOD and soluble guaiacol peroxidase) in plants leaves under the normal growth conditions. Low temperature exposure (5°C, photoperiod 24 h, irradiance of 180–200 mmol/m²·s, 7 days) resulted in the activation of respiration, synthesis of dehydroascorbate, increase of soluble carbohydrates, proline and anthocyanins contents in the leaves plants of all lines. The accumulation of carbohydrates and anthocyanins was the highest in the XX-2 line and proline – in the XX-2 and AS-12 lines. The increase of the respiration was the highest in Col-0 and the lowest in AS-12. The significant SOD activation and the decrease of superoxide radical ion generation were observed in the AS-12 line. Physiological and biochemical changes during the low temperature exposure led to an increase in Arabidopsis frost resistance. Frost resistance as in the control and hardened plants was higher in the AS-12 and XX-2 lines, but the productivity of seeds was higher in the XX-2 line. Thus, changes in the expression of Arabidopsis Aox1a cause significant restructuring in the metabolism of cells and determines the development of frost resistance.

**SW06.S27–29**
The SWI/SNF complex in Arabidopsis responds to environmental changes in temperature – dependent manner

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The SWI/SNF chromatin remodeling complexes (CRCs) have been shown to play important roles in regulation of gene expression throughout eukaryotes.

The Arabidopsis genome encodes four SWI2/SNF2 ATPases (BRAHMA, CHR12, CHR23 and SPLAYED), four members of SWI3 subunit (ATSWI3A, ATSWI3B, ATSWI3C, ATSWI3D), a single SNF5 and two ATSWP73 subunits. Most of the genes encoding these core components of Arabidopsis SWI/SNF CRCs have critical but not fully overlapping roles during plant growth, including embryo- and sporophyte development.

During our study using qPCR and promoter:GUS fusions we found that genes encoding the SWI1/SNF CRC subunits are ubiquitously expressed and that their expression levels depend on the temperature regime of growth. Furthermore, Arabidopsis mutants impaired in several of these genes growing at lower tem-
peratures show partial alleviation of their phenotypic defects, including reduced fertility, root development, and others.

In summary, our data provide novel insight into potential regulatory role of SWI/SNF CRCs during plant growth at different temperature ranges.

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Abstracts

SW06.S27–30
The evaluation of the new potato lines' drought resistance by peroxidase and superoxide dismutase enzyme activity
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Peroxidation enzymes – superoxide dismutase (SOD) and peroxidase – are components of plants' antioxidant protection system; they participate in regulation of active oxygen forms, which are formed in response to various stress factors. Level of these enzymes' activity in plant cells after such unfavorable factors as drought and temperature increase may serve as one of reliable criteria in evaluation of potato new lines' drought resistance. The goal of the research was to study activity changes in antioxidant enzymes superoxide dismutase and peroxidase in selective cell cultures and regenerant plants of potato in the conditions of drought and high temperature. Two genotypes with contrast resistance to drought were used in the research: drought-resistant potato variety Aksor and not resistant variety Baksha.

Cell cultures and regenerant plants with increased resistance to artificial osmotic stress of both potato varieties were obtained by cell selection method on the media with 0.15 M mannitol to imitate water deficiency. Enzyme activity of superoxide dismutase and peroxidase was analyzed on selective and control 9-days suspension cultures and regenerant potato plants of two contrast selective lines R37/A (Aksor) and R 4/B (Baksha). Samples were treated with high temperature (35°C) and 0.15 M mannitol as osmotic agent. Selection of the samples was carried out 2, 4, 8, 12, 24, 72, 168 and 288 h after stress factors were applied.

Biochemical analysis of superoxide dismutase and peroxidase enzyme activity revealed considerable difference in their activity levels in cell cultures and regenerant plants of selective and control potato forms.

It is established that in selective lines R37/A with high drought resistance level the activity of each antioxidant enzyme is much higher than in cell cultures and regenerant plants of selective line in contrast in drought resistance potato variety R 4/B. At the same time, in selective lines R37/A high temperature influenced the activity of superoxide dismutase and peroxidase more than osmotic agent.

It is shown that in suspension cells and regenerant plants of selective lines R37/A and R 4/B the level of superoxide dismutase and peroxidase activity was considerably higher in response to both temperature and osmotic stress factors (average increase in 1.5 times for peroxidase and 1.8 times for superoxide dismutase) than in suspensions and regenerant plants of original varieties.

Thus, the research results indicate that superoxide dismutase and peroxidase activities may serve as criteria for preliminary evaluation of new selective potato lines' drought resistance on cell level.
In order to improve the stability and quality of flax oil, three genetic modifications have been accomplished leading to the increase in phenolic compounds in seeds. In the first strategy, chalcone synthase (CHS) gene has been introduced to plants (W86 type), in the second strategy genes coding for chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) (W92 type) and in the third strategy gene coding for 7-O-glucosyltransferase (GT type). As a result, in all the three cases oils of higher resistance to oxidation have been obtained, as the levels of phenylpropanoids were elevated. Moreover, quantitative changes in fatty acids in W86 type led to an almost ideal α-6/α-3 ratio in oil.

The goal of the research was the determination of fatty acid composition, identification of water soluble compounds in oil from the transgenic types of flax in comparison to the non-transgenic control (Linola variation), as well as determination if the emulsions produced based on transgenic flax oils show protective and antioxidative properties towards fibroblasts and monocytes.

**SW06.S27–34**

Functional characterization of NADPH-cytochrome P450 reductase from hot pepper (*Capsicum annuum*)


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Cytochrome P450 (P450 or CYP) monooxygenases in plants have roles in the biosynthesis of plant hormones, defense related chemicals, and diverse secondary metabolites. We isolated cDNA for CYP707A70, which is encoded ABA 8'-hydroxylase from a cDNA library of hot pepper (*Capsicum annuum* L. cv. Bukang). The ABA 8'-hydroxylase is oxidative degradation of ABA to 8'-hydroxy ABA. Expression levels of CYP707A70 mRNA in various tissues were determined by quantitative-PCR analysis. The CYP707A70 expression level was increase after pollination and dramatically decreased after mature green stage. The CYP707A70 gene was transformed into tobacco (*Nicotiana tabacum* cv. Xanthi NC) and tomato (*Solanum lycopersicum* cv. Micro-Tom). Compared to wild type plants, the CYP707A70 over expressed transgenic plants showed more sensitive drought stress and reduced seed production and fruit enlargement. These results suggest that CYP707A70 is related to seed formation and fruit development.

**SW06.S27–35**

Functional characterization of CYP707A70 (ABA 8’-hydroxylases) from hot pepper (*Capsicum annuum*)

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Cytochrome P450 (P450 or CYP) monooxygenases in plants have roles in the biosynthesis of plant hormones, defense related chemicals, and diverse secondary metabolites. We isolated cDNA for CYP707A70, which is encoded ABA 8'-hydroxylase from a cDNA library of hot pepper (*Capsicum annuum* L. cv. Bukang). The ABA 8'-hydroxylase is oxidative degradation of ABA to 8'-hydroxy ABA. Expression levels of CYP707A70 mRNA in various tissues were determined by quantitative-PCR analysis. The CYP707A70 expression level was increase after pollination and dramatically decreased after mature green stage. The CYP707A70 gene was transformed into tobacco (*Nicotiana tabacum* cv. Xanthi NC) and tomato (*Solanum lycopersicum* cv. Micro-Tom). Compared to wild type plants, the CYP707A70 over expressed transgenic plants showed more sensitive drought stress and reduced seed production and fruit enlargement. These results suggest that CYP707A70 is related to seed formation and fruit development.

**SW06.S27–36**

Jasmonate-induced pea pod proteins

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Stress phytohormone jasmonic acid (JA) is a key factor of plant immunity against necrotrophic pathogens and some herbivores. During the infection with pathogens or wounding in plant tissues jasmonic acid is rapidly accumulated. The last ‘switching-on’ jasmonate dependent signaling pathway and activate synthesis of the proteins participating in plants defense reactions. Derivative of jasmonic acid methyl jasmonate (MeJ) also poses with signal functions. MeJ can be transported through the plant and induce systemic immunity. In our research we studied effect of exogenous MeJ on the induction of soluble proteins in plant roots using methods of proteomics (2DE and mass-spectrometry). We found that under the action of exogenous MeJ the content of 35 proteins increased and the content of 50 proteins decreased. Among the identified MeJ-induced proteins we found some marker proteins to the action of MeJ. These proteins are proteinase inhibitors and defensins. The decrease of the tubulin (component of cytoskeleton) can be connected with the plants growth inhibition under the action of JA and MeJ. Also incorporation of the 14C labeled amino acids in to the pea roots proteins was studied. It can help clarify the contribution of synthesis and degradation of proteins on the change of their content.

**SW06.S27–37**

Insights into the regulation of starch synthase activity from the crystal structure of barley starch synthase I

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Starch is the main storage carbohydrate in plants and the major source of calories in the human diet. In higher plants starch is synthesized in plastids in the form of starch granules which have
different sizes, shapes and degrees of crystallinity depending on botanical origin. Several classes of enzymes are involved in starch biosynthesis including ADP-glucose pyrophosphorylase, starch synthases, branching and debranching enzymes and phosphorylase. It is the coordinated action of all of these enzymes that ultimately controls starch structure and properties. There are five gene classes of starch synthases (SSs) that play different roles in elongation of the amylose and amylopectin chains, the two components that determine the physicochemical properties of starch. SS catalyzes the transfer of glucose from ADP-glucose to glucans forming α-1,4-glycosidic linkages. Details of their reaction mechanism are unclear, including how they select substrates for elongation, how they compete with each other and how their activities are regulated.

The crystal structure of barley starch synthase I (SSI) has been determined and refined to 2.7 Å resolution. The structure shows the characteristic glycosyltransferase GT-B fold for barley SSI in an open conformation with a surface-bound maltooligosaccharide (MOS). While the MOS-binding site is involved in substrate recognition, a disulfide bridge in the structure precludes the formation of the active site and suggests possible redox regulation of SSI which is supported by activity measurements on SSI mutants.

Plant SSIs from wheat, maize and barley possess an N-terminal extension with no predicted structure and function. The shorter rice counterpart lacks this extension and displays higher enzymatic activity. Truncated versions of barley SSI have enhanced catalytic activity, suggesting the N-terminal extension may have a regulatory role in substrate selectivity or coordination/interaction with protein partners. Our structure gives a better understanding of the effects mutations of SS will have on starch properties, which can guide starch bioengineering.

**SW06.S27–38**

*Ralstonia solanacearum* lectin elicits defense reaction in tobacco cells

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*Ralstonia solanacearum* is a plant pathogen inhabiting water and soil and causing lethal wilt in more than 200 plant species. These bacteria were shown to produce a potent l-fucose-binding lectin, *R. solanacearum* lectin (RSL), a small protein of 90 amino acids with a tandem repeat in its amino acid sequence. The biological target of the lectin may be xyleoglucan, a fucosylated polysaccharide from the primary cell wall of plants. This protein-carbohydrate interaction may be used by the bacteria for host recognition, attachment, and further invasion.

Plants have evolved various mechanisms to deal with pathogen attack. One of the key features of a plant’s defense against pathogens is the ability to discriminate between self and non-self. In plants, multiple defense responses are triggered by recognition of diverse molecules originating from pathogens, known as elicitors. Following elicitor perception, the activation of signal transduction pathways generally leads to the production of reactive oxygen species, phytoalexin biosynthesis, reinforcement of plant cell walls, and the accumulation of pathogenesis-related proteins, some of which possess antimicrobial properties.

In the present study, RSL protein is shown to elicit early defense reaction events in tobacco cell suspension cultures (*Nicotiana tabacum* L. cv. Xanthi). In RSL-treated tobacco cells, reactive oxygen species production and extracellular medium alkalization due to H+ flux modification are observed. These defense responses may, however, not be found in tobacco cells co-treated with RSL and fucose. These results suggest that specific binding of the RSL protein to fucosylated moieties present on the plant cell surface not the protein itself serves as a starting signal to plant response. This work was funded by the European Community’s Seventh Framework Programme under the European Regional Development Fund (CZ.1.05/1.1.00/02.0068). The presentation of this work was supported by the Czech Ministry of Education and by the European Social Fund in the Czech Republic (CZ.1.07/2.4.00/31.0133).

**SW06.S27–39**

Effect of sterol binding on biological activity of cryptogein in *Nicotiana tabacum*

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Cryptogein is a small (10 kDa) proteinaceous elicitor secreted by oomycete *Phytophthora cryptogea* which induces a defence reaction in *Nicotiana tabacum*. The elicitors consist of 98 amino acid residues with common structure comprised of five α-helices and one β-sheet arranged in a unique fold. In the protein core, there is a hydrophobic cavity which is connected to the protein surface by a tunnel. On the entrance to the cavity there is located a w-loop. In the tobacco plants, cryptogein induces a hypersensitivity-like response which is characterized by the induction of programmed cell death at the site of the infection and by the development of systemic acquired resistance at the entire plant. When applied to tobacco cell suspensions cryptogein triggers early events of the defence response including depolarization of the plasma membrane, potassium and chloride efflux, influx of calcium ions, alkalisation of the extracellular medium and production of active oxygen species (AOS) and nitric oxide. As a late process, the lipid peroxidation or the production of ethylene, phytoalexins and pathogenesis-related proteins were observed. Previous studies show that cryptogein together with other elicitors are a new class of sterol carrier proteins which are able to bind and transport sterols between biological membranes. Elicitins bind also fatty acids although this affinity is significantly lower. The sterol binding was assumed to be an essential step for cryptogein recognition by a plant receptor and for its biological activity in term of the induction of early events of defence reaction and the development of plant cell death. As well was found that sterol binding to the cavity result in a conformation change in the w-loop. For study of cryptogein biological activity a series of cryptogein mutants were constructed. A link between the changes of structure of cryptogein and the stimulation of the early events (AOS, pH and nitric oxide) in tobacco cells and also the induction of lipid peroxidation (necrotic effect) in tobacco plants was examined.

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A novel chloroplast localized Rab GTPase involved in stress, development, thylakoid biogenesis and vesicle transport in Arabidopsis

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Swertia chirata activity. Knockout mutants of sis. Overexpressed CPRabA5e showed intrinsic GTPase hydrolysis activity. CPRabA5e was detected in stroma and thylakoids in Arabidopsis. Transient expression of CPRabA5e:EGFP fusion protein in 4-week-old tobacco leaves revealed localization of CPRabA5e in chloroplasts, which also supported by immunoblotting where CPRabA5e was detected in stroma and thylakoids in Arabidopsis. Overexpressed CPRabA5e showed intrinsic GTPase hydrolysis activity. Knockout mutants of CPRabA5e exhibited delayed seed germination but otherwise no visible phenotypical variations under normal growth conditions compared to wild type. Furthermore, the mutants displayed growth arrest while exposed to oxidative stress using rose Bengal. A yeast-two-hybrid screen was performed and found that chloroplast S16 is derived from nuclear-encoded rps16. We first carried out the western blot analysis and found that chloroplast S16 is derived from nuclear-encoded rps16 gene. The RT-PCR analysis revealed that mRNA for chloroplast-encoded rps16 (cp-rps16) is accumulated at significant level. Then, we analyzed the translation regulation of cp-rps16 mRNA using chloroplast in vitro system. We found that cp-rps16 mRNA was not translated in spite of containing Shine-Dalgarno sequence at proper position. Further analysis using several chimeric mRNAs revealed that the 5' untranslated region of cp-rps16 mRNA causes the translation inactivation of this mRNA. Moreover, cp-rps16 mRNA has a translation activity in the reconstituted translation system from E. coli and this translation activity was reduced by the addition of chloroplast soluble fraction. These results suggest that chloroplasts have some factors to repress the translation of cp-rps16 mRNA.

Crocus sativus L. (saffron) has been traditionally used for the treatment of insomnia and other diseases of the nervous systems. Crocin is a digentiobiosyl ester of crocetin, an apocarotenoid aglycone, are major carotenoid pigment of saffron and a number of pharmacological studies have demonstrated that crocin and crocetin have a wide range of neuroprotective activities against Alzheimer’s disease, depression, and memory impairment. On the other hand, the effects of crocin and crocetin on sleep still remain unknown. In this study, we examined the sleep-promoting activity of crocin and crocetin by monitoring the locomotor activity and electroencephalogram after administration of these components to mice. Orally administered crocin (80 and 160 mg/kg of body weight) significantly suppressed the total amount of locomotor activity during the 12 h by 33% and 20%, respectively, as compared with the vehicle control. Crocin (30 and 100 mg/kg of body weight) increased the total time of non-rapid eye movement (non-REM) sleep by 60% and 170%, respectively, during a 4-h period from 20:00 to 24:00 after its intraperitoneal administration at a lights-off time of 20:00. Crocetin (100 mg/kg) also increased the total time of non-REM sleep by 50% after the administration. Compared with the vehicle-treated control, the number of non-REM sleep bouts increased by 2.2-fold and also those of wake bouts by 2.0-fold for 4 h after the crocin treatment. Crocin increased the number of stage transitions from wakefulness to non-REM sleep and from non-REM sleep to wakefulness by 110% and 190%, respectively. There was no significant difference in EEG power density of non-REM sleep between the crocin treatment and the vehicle control, indicating crocin did not affect the EEG power density of NREM sleep.

In addition, we enzymatically transglycosylated the crocin to improve the dissolution and absorption properties of poorly water-soluble crocin. Highly glycosylated crocin o showed 10–20-fold improvement in the absorption than crocin after oral administration.

We conclude that crocin is considered to induce non-REM sleep that is very similar to physiological sleep, suggesting its potential use for the treatment of insomnia.

The expression of the gene for chloroplast ribosomal protein S16 is translationally repressed in tobacco

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The chloroplast is the plant specific organelle derived from a cyanobacterial ancestor; therefore chloroplasts possess own genome and the gene expression system. During the chloroplast genome evolution, a large number of genes encoded by the ancestral chloroplast genome were transferred to the nuclear genome. The gene for chloroplast 30S ribosome subunit S16 (rps16) is quite unique, since rps16 is encoded not only by the chloroplast genome but also by the nuclear genome in many flowering plants. This raises the question of which gene, chloroplast or nuclear, is utilized to produce chloroplast S16. We first carried out the western blot analysis and found that chloroplast S16 is derived from nuclear-encoded rps16 gene. The RT-PCR analysis revealed that mRNA
Immobilization of Olive β-glucosidase on to superparamagnetic nanoparticles and its characterization

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Covalently binding of Olive β-glucosidase that active on the main olive phenolic glycosides, to superparamagnetic magnetite nanoparticles via carbodiimide activation was investigated and presented in this study. The properties of immobilized enzyme were investigated and compared to those of free enzyme. β-Glucosidase was purified from Edremit variety olive (Olea europea spp.) samples using ammonium sulfate precipitation and hydrophobic interaction chromatography (Sephrose 4B, 1-tyrosine, 1-Naphthylamine) and magnetic iron oxide nanoparticles were prepared by co-precipitation Fe3+ and Fe2+ ions in an ammonia solution at room temperature. Characterization of superparamagnetic particles was carried out by X-ray diffraction (XRD) and the magnetic measurements showed that the nanoparticles are magnetite and superparamagnetic, respectively. The immobilized enzyme showed higher activity than non-immobilized enzyme. The effects of various parameters such as pH, temperature, and storage stability on kinetic parameters of the immobilized enzyme immobilized enzyme were also investigated. Kinetic parameters of the immobilized enzyme were also evaluated. Thermal and storage stability experiments were carried out. It was observed that the immobilized enzyme had longer storage stability and retained 50% of its initial activity during 30 days.

Activities of alternative oxidase, uncoupling proteins and adenine nucleotide translocator in winter wheat mitochondria during cold hardening

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It is known that free fatty acids (FFA) uncouple oxidative phosphorylation and can inhibit the activity of alternative oxidase (AOX) in the plant mitochondria. Uncoupling by FFA may be related to the functioning of uncoupling proteins (UCPs) and adenine nucleotide translocator (ANT). AOX, UCPs and ANT are energy-dissipating systems of mitochondria and make an important contribution to the antioxidant defense of cells. The changes in the AOX, UCPs and ANT activity in the mitochondria from shoots of winter wheat etiolated seedlings, subjected to cold hardening (CH, 2°C, 7 days – I phase CH and –2°C, 2 days – II phase CH) have been studied. The FFA content has been regulated by the addition of bovine serum albumin (BSA) in the isolation and incubation mediums. Linoleic (LA) and palmitic (PA) acids and ATP (GDP) and carboxyatractyloside have been used to activate or inhibit UCPs and ANT, respectively.

It has been shown that the mitochondria oxidized exogenous NAD(P)H most intensively, then succinate, and the lowest rate was during malate oxidation. The activity of AOX inversely related to the substrate oxidation rate: it was the highest during malate oxidation and the lowest during NAD(P)H oxidation. The relationship between the content of FFA and the AOX activity in the mitochondria has been shown. Addition of BSA in the isolation and incubation mediums of mitochondria resulted in AOX activation. AOX activity in mitochondria increased during I phase CH and decreased during II phase CH. Stimulation of non-phosphorylating respiration by the LA and PA was the highest during the malate oxidation. The lowest contribution to the LA-stimulated respiration was introduced by UCP, and to the PA-stimulated respiration – ANT. Stimulation of non-phosphorylating respiration by FFA increased during I and II phase CH, while in the first case, the largest contribution to FA-induced uncoupling was brought by ANT, and during II phase CH – by UCP. Thus, during the CH of winter wheat etiolated seedlings functioning of mitochondrial respiratory chain is regulated by FFA content and maintained by AOX, UCPs and ANT, joint or consequence actions of which can prevent excessive reactive oxygen species production, to maintain stable work of the respiratory chain and energy costs of cells.

Inhibition of SAH-hydrolase during tobacco seeds germination induced by treatment by DHPA leads to mitotically heritable DNA hypomethylation, ectopic expression of floral genes and floral whorl malformations

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Development is connected to epigenetic modulation, e.g. changes of chromatin methylation. Methyltransferases utilize S-adenosylmethionine (SAM) as a methyl group donor and produce S-adenosylhomocysteine (SAH) which compete with SAM and generally inhibits all SAM-dependent methyltransferases. Low intracellular SAH level is maintained by SAH-hydrolases. The SAH/SAM ratio is tightly controlled but may be modulated by inhibitors of either SAM-synthetase or SAH-hydrolase. To study consequences of SAH-hydrolase inhibition on plant development we applied 9-(S)-(2,3-dihydroxypropyl)-adenine (DHPA), on tobacco seeds during germination. The drug decreased global DNA methylation in a specific, concentration dependent manner and CpG hypomethylation was mitotically inherited in plants grown further without the drug. Hypomethylated plants showed pleiotropic developmental defects including smaller stature, altered leaves and flowers, and decreased apical dominance and fertility. Expression of the floral meristem (NFL) and floral organ (NTDEF, NTGLO, NAG1) identity genes was studied. In control plants, the genes were highly expressed in flower buds but not in other organs. In hypomethylated plants, we detected markedly increased levels of NTDEF, NTGLO and NAG1 transcripts in leaves. Since tobacco is allotetraploid plant formed by interspecific hybridization between diploid progenitors close to modern N. sylvestris and N. tomentosiformis, we also analyzed contribution of individual tobacco progenitor genes to total transcript levels. In tobacco flower buds, both maternal N. sylvestris and paternal N. tomentosiformis progenitor genes are transcribed. In leaves from highly hypomethylated tobacco plants, the transcript levels reached those found in control flower buds and transcription was released from both parental genes. We conclude that induced DNA hypomethylation can deregulate expression of floral genes (and perhaps other genes) later in development possibly contributing to aberrant flower phenotype. Germinating plants might be particularly sensitive to accurate setting of SAH/SAM levels.

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**SW06.S27–46**

**Protective effects of grape seed proanthocyanidin extract in experimental obstructive jaundice**

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Obstructive jaundice is one of the most important surgical causes of jaundices. In this experimental obstructive jaundice study, we aimed to investigate the possible protective effects of grape seed proanthocyanidin extract (GSPE) which is a powerful free radical scavenger and antioxidant.

Thirty Wistar-albino rats were randomly allocated into three groups as control, sham and treatment group (n = 10). Only laparotomy was performed in the sham group. Common bile duct ligation was performed in the non-treatment group. In the treatment group, common bile duct ligation was followed by oral administration of 100 mg/kg/day doses of GSPE extract for 10 days from post operative first day. 5 cc blood samples and liver were harvested in order to evaluate the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), total bilirubin (T.Bil), direct bilirubin (D.Bil), malondialdehyde (MDA), total antioxidant status (TAS) and total oxidant status (TOS). Results were analyzed by Kruskal–Wallis Test and Mann–Whitney U test statistically.

Movements were slowed down and weight loss was observed in rats with obstructive jaundice groups. Two rats of sham group and three rats of other each groups died during the experimental period due to anesthesia, laparotomy, etc. There was a statistically significant difference in all parameters (p < 0.05) between the sham group and other groups. However, there was no difference between treatment and non-treatment groups (p > 0.05).

There is no evidence for protective effects of GSPE on organ damage in experimental obstructive jaundice with these findings. More comprehensive and comparative, experimental and clinical studies should be performed in order to find beneficial effect of Proanthocyanidin for the treatment of organ failure due to biliary obstruction.

**SW06.S27–47**

**Antielastase and antityrosinase activities of some thiocarbohydrazones Schiff bases**

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Thiocarbohydrazides constitute an important class of N, S donor ligands possessing interesting chemical, biological and medicinal properties. Both hydrazine groups of thiocarbohydrazide are very reactive and predominantly form bis thiocarbohydrazone derivatives with aldehydes and ketones.

Thiocarbohydrazones have attracted much attention due to their several pharmacological activities. So far, no report is available on the antielastase and antityrosinase activities of thiocarbohydrazones Schiff bases examined in this study.

Thiocarbohydrazide was prepared according by the reaction of carbon disulfide with hydrazine hydrate. Thiocarbohydrazone Schiff base ligands (L1, L2, L3) were obtained by the condensation of thiocarbohydrazide with carbonyl compounds such as isatin, 2-hydroxy benzaldehyde, 2-hydroxy naphthaldehyde respectively, according to the methods reported previously.

All of the tested compounds exhibited antielastase and antityrosinase activities. The inhibition was increased with increasing thiocarbohydrazone Schiff base’s concentration.

L1 compound showed the highest antielastase activity (IC50 = 1.1 10^-7 mg/ml). The antielastase activity is decreasing as L1>L2>L3. L1 compound showed also the highest antityrosinase activity (IC50 = 3.13.10^-5 mg/ml). The antityrosinase activity is decreasing in the following order; L1>L2>L3.

The results showed that L1 had effective antielastase and antityrosinase activities. These compounds could be used as a source of antielastase and antityrosinase activities in cosmetic and pharmaceutical industries.
SW06.S27–50
The influence of mitochondrial pore modulator (Cyclosporine A) on oxidative processes in the organs of heat-treated wheat seedlings 

Triticum aestivum L.

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Cyclosporine A (CsA) prevents opening of mitochondrial permeability transition pore and therefore CsA can delay oxidative processes. The influence of CsA on oxidative processes in etiolated wheat seedlings under heat stress (HS) (42°C) at the early stage of seedling development was studied. The 4-day-old seedlings were exposed by short-term (15, 30 and 60 min) and long-term (24 h) HS. Samples (first leaves, coleoptiles) were taken immediately after HS exposure and at 24-h intervals for measurement of lipid peroxidation product (malondialdehyde, MDA), electrolyte leakage (EL), changes of total soluble protein amount and electrophoretic activity of antioxidant enzymes (CAT, SOD, POD).

The CsA induced decrease of lipid peroxidation increased membrane stability of the first leaves and roots, but membrane stability of the coleoptiles was similar to control. However, HS exposure changed the influence of CsA on mitochondrial pores, and, as a result, it changed oxidative processes in the organs of wheat seedlings. The presence of CsA significantly decreased the EL in the first leaves and coleoptiles after the 15- and 30-min HS exposure. But, 60-min and 24-h HS destroyed the inhibiting influence of CsA immediately after stress exposure and the EL increased significantly. However, the first leaves were able to decrease membrane permeability after high temperature induced oxidative stress. The short-term HS and CsA complex provoked the inhibition of lipid peroxidation, increased the electrophoretic activity of SOD and CAT in the first leaves.

There by the presence of CsA stabilizes the membrane lipids of the first leaves of wheat seedlings and decreases oxidative processes, which is induced by short-term heat stress. However, long-term HS do not change the sensitivity of mitochondrial pore against CsA in the first leaves; bet reduced the influence of CsA in the roots. The sensitivity of mitochondrial pore against influence of CsA may be organs specific.

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SW06.S27–51
The influence of inhibition of electronic transport by antimycin A on oxidative and photosyntetic processes in some organs of wheat seedlings

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The interaction between cell respiration and photosynthesis is important process in plant biochemistry metabolisms. Antimycin A (AA) is a potent electron transport chain (ETC) inhibitor, which inhibits the cyclic electron transport in chloroplasts and the flow of electrons through complex III of the ETC by blocking the passage of electrons from cytochrome b to cytochrome c in mitochondria. The influence of antimycin A on some oxidative and photosyntetic processes in functional different organs (coleoptiles and first leaves) of winter wheat seedlings Triticum aestivum L. was studied. The wheat seedlings were growth on antimycin A solution (1 mg/l) in dark and light conditions in climate camera during early stages (from 3 to 7 day). The superoxide radical (O2−) production, lipid peroxidation product (malondialdehyde, MDA), pigments concentration (chlorophyll a, b and carotenoids) and chlorophyll fluorescence (Fm-F0/Fm = Fv/m.) were measured. It was shown, that under influence of antimycin A the rate of (O2−) production and MDA concentration changes at division and not division cell populations of coleoptiles and first leaves on different stages of wheat seedlings development in dark and light conditions. The concentration of chlorophyll a, b is decreased under influence of antimycin A, bet the concentration of carotenoids is increased. We observed a sensitive of photosynthesis to antimycin A (monitored by chlorophyll fluorescence).

It was assumed to the existence of two different of cyclic electron flow in plant cells: antimycin A-sensitive and NDH-dependent.

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SW06.S27–52
Evaluation of Raman spectroscopy for investigation of DNA methylation in plants

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The changes of methylation degree in nuclear DNA that involve with hypomethylation and hypermethylation are frequently observed in normal and pathological cellular processes, contributing to development and differentiation in plants, animals and fungi. In nowadays DNA methylation also apply for diagnostic of physiological and pathological process. There are many methods for investigation of DNA methylation, for example: restriction analysis, pyrosequencing and others. But these methods are relative expensive. In this research we evaluated Raman spectroscopy for DNA methylation studies in wheat (Triticum aestivum L). We investigated DNA from wheat seedlings, which grow in water and DNA from wheat seedlings, which grow in solution with 5-azacytidine. 5-azacytidine inhibits nuclear DNA methylation. For DNA extraction Qiagen DNeasy plant kit was used. The nuclear DNA methylation level was checked out by pyrosequencing with Pyromax Q24 system. It was the first attempt to quantitatively analyze plant global genomic DNA methylation, using a luminometric technology compiled with methylation sensitive restriction analysis.

The Raman spectroscopy can provide information about chemical composition of the investigated samples, also applying...
for DNA analysis. A Raman spectrum shows many structurally sensitive Raman bands. Compared to other molecular techniques what are used for DNA methylation investigation, one of the advantages of Raman spectroscopy is research without reagents. The Raman spectra were collected with Raman inv/ia Reflex microscopy system in range at 500/cm to 1750/cm with Renishaw NIR 785 nm diode laser and 514.5 nm argon ion laser. Various bands in the range at 1200/cm to 1600/cm region assigned to nucleotide ring vibration are sensitive to cytosine methylation with 5-methylcytosine formation. A detailed analysis of the Raman shifts and the intensity dynamics of the peak will be able to expand the application of Raman spectroscopy in the expression-diagnostic research of DNA methylation.

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**SW06.S27–53**  
Isolation of oil-degrading microorganisms from rhizosphere and rhizosphere of plants and evaluation of their destructive activity

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Oil and soil products are the most common pollutants of the environment. In addition to the physical and chemical methods of cleaning contaminated soil rhizoremediation as a method of biological treatment attracts specialists. Rhizoremediation based on the use of micro-organisms capable of utilizing petroleum hydrocarbons as the sole carbon and energy source, and includes the destruction of organic pollutants associated with plant microorganisms. Normally, this process involves microorganisms of rhizosphere and rhizosphere zones of plants and mycorrhizal fungi.

Plants, pre-selected by their ability to grow on contaminated soils, alfalfa, grass mixture, and barley were used. Cultivation of microorganisms to account for hydrocarbon-oxidizing bacteria was carried out in a liquid synthetic medium SE; oil was used as a source of carbon and energy used. Assessment of the destructive activity of cultures was identified visually by destroying of the oil film on the 3 and 5 days of culture at the oil concentration of 2 g/l and 5 g/l of medium.

It has been shown that the number of oil-degrading microorganisms in the rhizosphere and rhizosphere of all plants increased hundreds of times compared to the number of them with soil. In addition, the number of oil-degrading microorganisms in rhizosphere was higher compared to the rhizosphere. So, the number of alfalfa in the rhizosphere was 956 \( 9.5 \times 10^3 \) CFU/g of soil, and in rhizosphere was higher and amounted to 4070 \( 5.0 \times 10^3 \) CFU/g of soil.

Four hundred and twenty cultures of microorganisms by their ability to grow in a medium containing oil at a concentration of 2 g/l of medium were allocated. 300 cultures were from rhizosphere of plants and 120 - from the rhizosphere.

Destructive activity by their ability to grow in a medium containing oil in concentration of 2 g/l was estimated through selected cultures of microorganisms. 240 isolates for which the degree of degradation of oil ranged from 60 to 85% were selected.

When the oil concentration was 5 g per litre, rate of destruction ranged from 34 to 62%, which was carried out by 35% of the selected isolates.

**SW06.S28 Glycobiology: Carbohydrate-Protein Recognition (VI-S28)**

**SW06.S28–1**  
Recognizing sugars: Identification of glycan-binding receptors in innate and adaptive immunity

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Receptors containing calcium-dependent carbohydrate-recognition domains (C-type CRDs) play multiple roles in the immune system. Receptors found on macrophages, dendritic cells and endothelial cells in the innate immune system often bind broad classes of sugar structures, facilitating selective interaction with viral, bacterial and fungal pathogens. Some of these receptors, such as DC-SIGN and the macrophage galactose receptor, were isolated based on their biological functions, while others such as prolein and minkle have been identified by screening of the human genome. In both cases, a key question about these receptors is how they bind selectively to appropriate targets. This point has been addressed by examining different ways that simple primary monosaccharide-binding sites in these receptors are extended with secondary binding sites that create specificity for oligosaccharides and the way that the binding sites are clustered in receptor oligomers to facilitate recognition of pathogen surfaces. A second key issue is what happens when the ligands are bound. Many of the receptors mediate pathogen clearance and antigen presentation by functioning in receptor-mediated endocytosis. However, there is increasing interest in the less well understood ways in which glycan-binding receptors initiate signaling cascades. The way in which the two processes of glycan target recognition and functional outcomes are linked is being explored through structural analysis of the receptors and their complexes with glycan ligands.

**SW06.S28–2**  
How human lectins translate the sugar code

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Biochemists would miss intriguing insights into an emerging dimension of molecular functionality, if they do not take note that glycosylation means much more than just modifying a protein’s structure. In fact, glycans are the chemical platform of the sugar code [1]. These chains of cellular glycoconjugates (glycoproteins, glycolipids) are not only known to be phenotypic markers. As product of a fine-tuned synthetic machinery, distinct determinants can serve as molecular messages decoded into physiological effects by endogenous sugar receptors (lectins) [1,2]. Underscoring the versatility of sugar coding, at least 14 different protein folds have developed in animals and man with the capacity to bind glycans, among them the b-sandwich [1,2]. Members of a lectin family with this folding pattern, sharing binding of b-galactosides on cell surfaces only few types of glycoconjugates are destined to become functional counterreceptors (e.g. ganglioside GM1 or the a,b3-integrin). This recognition process starts the cascade of post-binding events toward effector mechanisms (e.g. caspase-8 activation or Ca\(^{2+}\) influx) [3]. The case of the tumor suppressor p16\(^{DNK44}\) teaches us the lesson how orchestrated regulation of glycoenzymes (glycosyltransferases, enzymes
of sugar synthesis), cell surface glycosylation and galectin expression leads to anoikis induction in pancreas carcinoma cells in vitro, a potential functional link between suppressor integrity and tumor incidence. A key open question is to identify the molecular mechanisms how tissue lectins find their matching binding partner(s), amidst the complexity of the glycome. Analysis of lectin specificity using cell systems is a salient step toward this aim. Experimental data guide to a system on six levels to explain the exquisitely specific selection process, turning a certain glycoconjugate into the binding partner. Evidently, structure and topology matter, on both sides, to translate sugar-encoded information into cell-type-specific responses.

**References**


**SW06.S28-3**

**Structure, function and evolution of retaining glycosyltransferases**

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Glycosyltransferases catalyze the synthesis of glycosidic linkages by transfer of a sugar from a donor to an acceptor substrate. Glycosyltransferase reactions are, with few exceptions, regiospecific and stereospecific and reaction occurs with either retention or inversion of the anomeric configuration of the transferred sugar. The most common donors are nucleotide-sugars while acceptors are saccharides, proteins, nucleic acids, and lipids, natural and unnatural products. Currently over 110 000 ORFs for glycosyltransferases are tabulated in 94 families in the CAZy database corresponding to about 1% of the genes of all genomes sequenced to date. Over 90% of the ORFs are uncharacterized thus biochemical validation is a major endeavor. X-ray structural studies of over 100 nucleotide sugar utilizing glycosyltransferases in 38 families have revealed surprisingly conserved architecture with the observation of only two types of structural fold, or variants thereof, termed the GT-A and GT-B fold.

We have been using the blood group A and B synthesizing glycosyltransferases (GTA and GTB) members of CAZy family 6 as structural and mechanistic models for retaining enzymes with the GT-A fold. In December 2012, GTA and GTB were selected as molecules of the month by PDB. GTA and GTB catalyze the transfer of GalNAc or Gal, respectively, from their corresponding UDP-sugar donors to a common H-disaccharide motif (alphaFuc(1,2)betaGal-R). The enzymes differ by only four critical amino acids of 354; Arg/Gly176, Gly/Ser235, Leu/Met266 and Gly/Ala268 in GTA and GTB. Recombinant enzymes with interchanges of the critical residues have revealed the contribution of each residue in substrate discrimination and turnover. Mutagenesis of the four critical and/or non-critical amino acids can generate rare cis-AB enzymes where a single enzyme can catalyze both GTA and GTB reactions. The X-ray structures of wild-type enzymes and their complexes with substrate suggest they undergo numerous conformational changes upon substrate binding and throughout the catalytic cycle. These can be exploited in the design of inhibitors that interfere with loop reorganization. Mutants discovered in blood banking laboratories have revealed the surprising resiliency of GTA and GTB to mutagenesis.

**SW06.S28-4**

**Selectin antagonists as anti-inflammatory agents: a glycomimetic approach**

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The selectins play a key role in the body’s defense mechanism against inflammation. They form a class of three cell adhesion molecules (E-, P-, and L-selectin), which, in case of an inflammatory stimulus, are responsible for the initial steps of the inflammatory response, that is, the tethering and rolling of leukocytes on the endothelial surface of blood vessels. These steps are a prerequisite for the subsequent firm adhesion and the final extravasation of leukocytes to the site of the inflammatory stimulus. However, excessive infiltration of leukocytes into the adjacent tissue can lead to acute and chronic reactions, as observed in reperfusion injuries, stroke or rheumatoid arthritis. Therefore, the antagonism of selectins is regarded as a valuable pharmaceutical option.

The physiological selectin ligands are glycoproteins. Their KDs for the interaction with selectins are generally micromolar and only in one case (PSGL-1/P-selectin) nanomolar. Glycosidase treatment reduces, but not abolishes, their affinity, indicating that both, their carbohydrate as well as their peptidic part, contribute to binding. Therefore, we envisaged a dual approach, aiming at the identification of mimetics of the carbohydrate epitope as well as the peptidic part. By linking these two fragments, high affinity ligands are aspired.

The tetrascaccharide sLe^a, although exhibiting only a moderate binding affinity (K_D = 877 μM for E-selectin), was chosen as starting point in our search for high-affinity glycomimetics. Furthermore, the characteristics of their interaction with E-selectin, i.e. their binding thermodynamics and kinetics were determined by isothermal titration calorimetry and surface plasmon resonance. In the second part, two different fragment-based approaches leading to the identification of mimetics of the peptidic contribution are discussed. As a result, novel E-selectin antagonists and a pan-selectin antagonist (successfully evaluated in phase II clinical trials) were identified.

**References**

**SW06.S28-5**
**Histo-blood group antigens in host-pathogens co-evolution and providers of «Herd Innate Protection»**

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Many caliciviruses of the norovirus and lagovirus genus (RHDV) bind to HBGAs, which show a polymorphism within the host species. The strains of norovirus that bind to HBGAs can collectively infect all humans, causing acute gastroenteritis, but each strain infects a subgroup of the population only, suggesting a past co-evolution of humans and noroviruses that led to a trade-off where the human population is partly protected whilst the virus circulation is maintained. We termed «Herd Innate Protection» the partial protection provided by the HBGAs polymorphism. Given its recent emergence, its high virulence and its ability to bind to HBGAs, RHDV is expected to exert a strong selective pressure on some glycosyltransferase genes of rabbits, providing a model suitable for studying calicivirus-host-co-evolution based on field observations. Our recent results using this host-pathogen pair provide evidence for evolution of the virus ability to recognize the host HBGA diversity and for strain-host-pathogen pair provide evidence for evolution of the virus ability to recognize the host HBGA diversity and for strain-dependent selection at the α1,2-fucosyltransferases locus and of ABO phenotypes following outbreaks. This indicates that a host-pathogen co-evolution involving HBGA recognition is indeed taking place. The model can be used to explain the epidemiology of human noroviruses and recent data suggest that it may be extended to human rotaviruses.

**SW06.S28-6**
**Siglecs mediate B cell tolerance as sensors of self**

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The sialic acid binding immunoglobulin lectin (siglec) family of cell adhesion molecules are differentially expressed on white blood cells that confer innate and adaptive immune responses. Because they recognize sialic acid containing glycans as ligands, which are expressed on all mammalian cells, the siglecs are increasingly recognized as receptors that help the immune system distinguish between self and non-self. Exemplary are the B cell siglecs CD22 and Siglec-10 (Siglec-G in mouse), which are well distinguished in negative regulation of the B cell receptor. We have obtained evidence to support the view that B cell siglecs help enforce B cell tolerance to self-membrane antigens. When a B cell recognizes an antigen on a cell that contains siglec ligands, the siglecs are recruited to the site of the immunological synapse, resulting in suppression of B cell signaling. Moreover, the impact of the siglec is to suppress the Akt survival pathway, resulting in apoptosis of the antigen reactive cell. We have also found that this natural regulatory mechanism can be exploited to induce tolerance to any desired antigen. To this end, we have developed liposomal nanoparticles bearing a multivalent display of an antigen and high avidity siglec ligands that can target antigen reactive cells in vivo. Remarkably, injection of the antigenic liposomes into mice results in induction of apoptosis in reactive B cells, and the mice are then incapable of mounting an antibody response to that antigen in a subsequent challenge. Since development of inhibitory antibodies to FVIII is a serious problem in treatment of hemophilia A patients, we investigated the potential of this approach for inducing tolerance to FVIII in a hemophilia mouse model. Our tolerizing liposomes prevented formation of inhibitory FVIII antibodies, allowing for effective administration of FVIII to hemophilia mice to prevent bleeding. Thus, we suggest that a major function of the B cell siglecs is to recognize sialic acid as ‘self’, and to induce apoptosis in autoreactive B cells for maintenance of peripheral tolerance. Exploiting this mechanism has therapeutic potential in the areas of autoimmunity, allergies, and biotherapeutics (NIH grants AI050143, AI099141, CA013889 and HFSP Fellowship LT001099/2010-L).

**SW06.S28-7**
**Plant cell wall polysaccharides: Interactions and supramolecular organization**

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Plant polysaccharides comprise the major portion of organic matter in the biosphere. These complex structures are built from a set of neutral and acidic monomers and may have the degree of polymerization in the range of many thousands. Around 10 types of polysaccharide backbones present in cell walls of higher plants can be decorated by different substituents giving rise to the endless diversity of carbohydrate structures. A plant uses the diversity of cell wall polysaccharides to perform the tissue- and stage-specific function. Identification of structure-function relationship for plant cell wall polysaccharides faces the general problems to study large polymers with non-template mode of synthesis combined with the difficulties to study the supramolecular organization of complex irregular structures. The structure of polysaccharides is not directly encoded by the genome and has the variability in many parameters (molecular weight, length and location of side chains, presence of modifying groups, etc.). The extent of such variability is limited by the “functional fitting” of the polymer, which is largely based on spatial organization of the polysaccharide and its ability to form supramolecular complexes of an appropriate type. The presentation summarizes the data on structural peculiarities of plant cell wall polysaccharides, considers formation of supramolecular complexes, gives the examples of tissue- and stage-specific polysaccharides and functionally significant carbohydrate-carbohydrate interactions in plant cell wall, and presents the approaches to analyze the spatial structure of polysaccharides and their complexes.

**SW06.S28-8**
**Cell landscape engineering**

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The ability to harmlessly modify the surface of any cell, with virtually any bioactive compound, has a number of potential in vitro and in vivo opportunities, ranging from cell targeting through to imaging. Recently a series of Function-Spacer-Lipid constructs...
When antibodies meet carbohydrates in blood

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A wide variety of so-called natural antibodies (nAbs), i.e. immunoglobulins generated by B-1 cells, is directed to glycans. nAbs to glycans can be divided in three groups: (i) conservative nAbs, i.e. practically the same in all healthy donors in respect of their epitope specificity and level in blood; (ii) allo-antibodies to blood group antigens; (iii) plastic antibodies related to the first or the second group but discussed separately because their level changes considerably during diseases and some temporary conditions, in particular inflammation and pregnancy. Antibodies from the third group proved to be prospective markers of a number of diseases, whereas their unusual level (below or above the norm) is not necessarily the consequence of disease/state. Modern glycan microarrays allowed the determination of repertoire for human nAbs which proved to be unexpectedly broad. It was observed that the content of some nAbs reaches about 0.1% of total immunoglobulins. IgMs dominate for most nAbs, constituting up to 80–90%. Their affinity (to a monovalent glycan, in K0 terms) found to be within the range 10^4 – 10^8 M. Antibodies to Gal(b)1-3GlcNAc (Le\(^\alpha\)), 4-HSO3Gal(b)1-4GalNAc (4'-O-SuLN), Fuc(a)1-3GlcNAc, Fuc(a)1-4GlcNAc, GalNAc(a)1-3Gal (Adi), Gal(a)1-3Gal(b)1-4GlcNAc, GlcNAc(a)-terminated glycans, and hyaluronic acid may be the target of the nAbs.

Renal glycosphingolipid Gb3Cer/CD77 expression in rat models of type 1 and type 2 diabetes

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Introduction: Considering diabetic nephropathy and changed glycosphingolipid metabolism in diabetes, the aim of this study was to determine glycosphingolipid globotriaosylceramide (Gb3Cer or CD77) expression on renal endothelial cells, in rat models of type 1 (T1DM) and type 2 diabetes mellitus (T2DM).

Materials and Methods: Diabetes was induced with streptozotocin (55 mg/kg for T1DM and 35 mg/kg for T2DM) injection to rats which were fed with normal pellet diet (T1DM, n = 14) or high-fat diet (T2DM, n = 10). The endothelial marker CD34 and glycosphingolipid CD77 were determined in renal cell suspensions by flow cytometry.

Results: Rats of T2DM model showed twice lower (0.44 ± 0.18 vs. 0.92 ± 0.64, mean ± SD, p = 0.016) proportion of renal CD77 positive non-endothelial cells in comparison to corresponding control rats.

Conclusion: Our study showed that the CD77 positive non-endothelial renal cells could specifically mirror diabetic nephropathy.
Glycosylation of envelope proteins of hepatitis C virus and their effects on the formation of virus particles


The details of the HCV particle structure and its assembly remain poorly explored, the processes of virion assembly and virus release from the cell being the least studied. The virion properties are reasonably believed to depend on the glycosylation of HCV envelope proteins in the infected cell, their interactions, and the type of folding.

Introduction: Study of conformational changes and interactions between HCV envelope proteins and their glycosylation in model systems based on insect and mammalian cells and the formation of self-assembling, nonreplicating particles and morphologically similar to native virions will enable to explore the role of structural HCV proteins in the morphogenesis viral cycle.

Methods: The effects of N-linked glycosylation of HCV protein E1 on the assembly of structural proteins were studied using site-directed mutagenesis and new inhibitors of glycosylation in a model system of Sf9 insect cells producing three viral structural proteins with the formation of virus-like particles due to the baculovirus expression system. This allows to define the effect of gpE1 and gpE2 on formation of glycoprotein complex, and consequently, on VLP assembly, and to provide the selection of inhibitors which most effectively block correct assembly of virus-like particles.

Results: Our results show that the absence of the carbohydrate chains of glycoproteins glycosylation sites in HCV, particularly in the E1N1 (196 aa.), E1N5 (305 aa.), E2N1 (417 aa.), E2N2 (423 aa.) and E2N9 (623 aa.), leads to formation of unproductive dimers E1E2 in insect Sf9 cells and mammalian Huh7 cells. Glycans in these sites are important for the formation of non-covalent, productive E1E2 complex and formation of VLP in Sf9 insect. It was shown that NΦo-DNJ and NΦo-DNJ in concentrations of 500 μM and 1 mM do affect glycosylation or the folding of virus envelope proteins by preventing gpE1E2 heterodimerization. Inhibition of α-glucosidase results in accumulation of uncompleted triglycosylated N-glycans that gives rise to nonproductive E1E2 dimers and thus suppresses productive VLP assembly in insect cells.

Conclusion: Using a model of HPV HCV opens up new possibilities for the study of viral morphogenesis and virus-cell interactions for immunological and therapeutic studies. N-linked glycans of glycoproteins gpE1 gpE2 HCV in the assembly process influence the formation of virus particles having an effect on virus infectivity. Destruction of glycosylation sites of these proteins leads to their misfolding and, apparently, the accumulation of unproductive dimer consisting of E1E2 VLP HCV. This may entail the formation of defective HPV different from natural, not capable of binding to the cell.

The studied α-glucosidase inhibitors, which suppress productive assembly VLP in cells and interfere with the key stages of HCV morphogenesis, can be used to develop a new generation of antiviral drugs.

Investigation of the mycobacterial ABC transporter involved in the assembly of the cell wall polysaccharides

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The ABC (ATP-binding cassette) transporters represent a large superfamily of integral membrane proteins. They utilize the energy from ATP hydrolysis into trans-bilayer movement of substrates. ATP hydrolysis is carried out by a pair of nucleotide-binding domains (NBDs), whereas the translocation of the substrate is facilitated by a pair of transmembrane domains (TMDs).

Our work is aimed at the investigation of the ABC transporter (Rv3781c/Rv3783c) from Mycobacterium tuberculosis – the causative agent of tuberculosis, which still kills about two million people every year. In silico analysis revealed that the genes encoding ABC transporters occupy about 2, 5% of the genome of M. tuberculosis H37Rv and at least 26 complete ABC transporters have been identified; however, so far none of them was sufficiently characterized. The participation of the ABC transporters in the phenomenon of drug resistance or their role in many essential bacterial pathways justify the efforts to study mycobacterial ABC transporters.

The transporter (Rv3781c/Rv3783c) is the only mycobacterial candidate implied in the transport of the polysaccharides to the cell surface. Several lines of evidence suggest its possible role in the biosynthesis of arabinogalactan, the major polysaccharide of mycobacterial cell wall. The peculiar cell wall is one of the key factors contributing to the pathogenicity of M. tuberculosis and also it is a validated target of some antituberculosis drugs. Here we provide in vitro characterization of the nucleotide-binding domain Rv3781c focused on the investigation of its ATPase activity, as well as the application of bacterial two hybrid system as the means of searching for the interaction partners of this transporter.

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Changes in the structure of leukocyte surface glycoconjugates in streptozotocin-induced diabetic rats and after treatment with agmatine

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Changes in the structure of leukocyte surface glycoconjugates were determined colorometrically using theperiodate-thiobarbituric acid assay. The change in expression of alpha2,3- and alpha2,6-linked sialic acids was determined by lectin-induced leukocyte aggregation analysis with lectins MAA and SNA. To further define the glycosylation
of cell surface glycoconjugates, leukocytes were analysed by enzyme-linked lectin assays (ELLA). The following lectins were used: RCA (for the identification of terminal Gal in the disaccharide Gal(beta1,4)GlcNAc), PNA (terminal Gal in the disaccharide Gal(beta1,3)GlcNAc), SBA (terminal GalNAc), LCA (bi- and triantennary complex-type N-glycans) and PHA (tri- and tetraantennary complex-type N-glycans).

The obtained data demonstrate an increase of desialylation of carbohydrate determinants of leukocyte membrane glycoconjugates in diabetic rats. This was associated with a decrease in both alpha2,3- and alpha2,6-linked sialic acids, uncovering the penultimate galactose residues and removal of O-linked oligosaccharides from the glycoprotein. These changes may be responsible for the modification of diverse cellular functions and change of signaling pathways in leukocytes. Our findings show increased sialation (in particular alpha2,3-linked sialic acids) of oligosaccharide sequences of leukocyte glycoconjugates after treatment with aminetine. It was also shown to enhance the content of O-glycans and decrease the exposure of terminal galactose residues of glycoconjugates. The detected changes in configuration of membrane components of leukocytes in diabetic animals after aminetine administration indicate the positive influence of treatment with this polyanine due to its hypoglycemic effect.

SW06.S28–15
Lectin binding infected with proteus vulgaris cell membranes spleen of rabbits
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In this study, Proteus vulgaris OX19 bacteria were injected by increasing doses (0.5 ml, 0.5 ml, 1 ml, 2 ml, 4 ml, 4 ml) in 5 days intervals to New Zealand adult male rabbits for 1 month. After 1 month from injection, spleen organs were taken from control group and infected group of rabbits. Sections of tissues were stained according to the method of Avidin-Biotin-Peroxidase with five lectins [Concavalla ensiformis (Con A), Arachis hipogaea agglutinin (PNA), Bauhinia purpurea (BPA), Griffonia simplicifolia (GS-I), Ulex europaeus agglutinin (UEA)] and examined by light microscopy. Among the used lectins, Con A was strongly stained (+ + +) for spleen cell membranes of Proteus infected group and moderately stained (+ +) for that of the control group. Also, UEA-I was weakly binding to control group cell membranes, on the other hand moderately binding to that of Proteus infected groups. PNA, BPA and GS-I lectins were strongly stained (+ + +) for spleen cell membranes of control group while that of the other group was weakly stained (+). According to the results of this study we can suggest that there are alterations in carbohydrate moieties of membrane glycoproteins and glycolipids of spleen cell membranes Proteus vulgaris OX19 infected groups rabbit with regard to that of control group rabbit.

SW06.S28–16
Gb3/CD77 synthase (alpha1,4-galactosyltransferase) and its variant form, NOR-synthase, exist as dimers
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Human Gb3/CD77 synthase (alpha1,4-galactosyltransferase; encoded by A4GALT gene) synthesizes the terminal Gal(alpha1-4)Gal moiety of globotriaosylceramide (Gb3/CD77, P8 antigen). The same enzyme, when upregulated, additionally synthesizes an identical moiety in PI glycosphingolipid belonging to neolecto series, whose presence or absence determines the P1 or P2 blood groups, respectively. Recently, a mutation C→G at the nucleotide position 631 of the A4GALT leading to a Q211E substitution in the enzyme, has been identified in the individuals possessing NOR antigens on red blood cells. NOR erythrocytes were found to contain globoside (Gb4) elongation products terminating with a unique Gal(alpha1-4)GalNAc sequence which is recognized by natural antibodies commonly present in human sera, and thus responsible for a rare type of inheritable polyagglutination (known as NOR polyagglutination). We found that the Q211E substitution alters the acceptor specificity of Gb3/CD77 synthase, rendering it able to catalyze the synthesis of both, Gal(alpha1-4)Gal and Gal(alpha1-4)GalNAc, moieties.

It has been shown recently that glycosyltransferases can form enzymatically active homo- and heterodimers. We hypothesized that the mutation found in A4GALT may cause Gb3/CD77 synthase to form an unusual heterodimer. In order to evaluate such possibility, we employed the bimolecular fluorescence complementation technique (BiFC); the DNA fragments encoding Gb3/CD77 synthase were cloned in-frame into pcDNA3 vectors harboring either N- or C-terminal fragments of the Venus variant of yellow fluorescent protein. When COS-7 cells were co-transfected with such vectors, complementation of Venus protein fragments gave a fluorescence signal, detectable in flow cytometry. We found that 57.4% of consensus and 35.7% of variant Gb3/CD77 synthase form dimers. Thus, we propose that the consensus Gb3/CD77 synthase exists as a dimer, while the Q211E substitution renders the enzyme more prone to form heterodimers at the cost of homodimerization.
synthase with Q211E substitution. Using the crystal violet staining assay, we found that susceptibility of the cells transfected with either vector was ten times higher than susceptibility of the untransfected cells. In the thin layer chromatography overlay of glycolipids derived from transfected 2102Ep cells, Shiga toxins were found to bind only to Gb3. TLC overlay of glycolipids from red blood cells also revealed the binding to Gb3, but in NOR-positive erythrocytes an additional band was detected, migrating similarly to P1 glycolipid. Thus, our results show that Shiga toxins VT1 and VT2 do not bind to NOR antigens suggesting that the penultimate galactose residue of the Shiga toxin receptor cannot be replaced by N-acetylgalactosamine.

**SW06.S28–18**

**X-ray structure of a stable protease-resistant galectin-9 with short linker**


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Human Galectin-9 (hG9) belongs to a lectin family and has two different carbohydrate recognition domains (N-terminal and C-terminal CRDs) which are specific to beta-galactosides. The physiological function of hG9 is miscellaneous and known to be disturbing their interaction.

**Reference**


**SW06.S28–19**

**A new lectin from coral *Gerardia savaglia*: purification, physico-chemical characterization and thermodynamics of saccharide binding**

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A new lectin has been purified from Mediterranean coral *G. savaglia* by affinity chromatography on mannose-sepharose. Two-dimension electrophoresis showed a single spot at pl 8.9, while MALDI TOF/TOF mass spectrometry analysis revealed four molecular variations with molecular weights 16 803, 16 769, 16 617 and 16 583 Da. Identical N-terminal amino acid sequence confirmed similarity between these four molecules. The hemaglutination activity of the lectin was analyzed at different temperatures, pH and in the presence of divalent cations. Far UV circular dichroism (CD) showed unusual spectra with high content of β-structures. Thermal stability was investigated by differential scanning calorimetry (DSC) and CD spectroscopy. The DSC yielded a curve with endothermic peak above 72°C with midpoint at 78.9°C, accompanied with loss of structure confirmed by CD. Both methods showed that the thermal unfolding process is irreversible. Lectin-D-mannose interactions were studied by CD and isothermal titration calorimetry (ITC). No changes in far UV CD spectra have been observed upon addition of mannose even in the presence of divalent cations. On the other hand ITC studies showed binding of mannose is accompanied by large negative enthalpy change (due to high number of hydrogen bonds) and unfavorable entropy contribution (due to solvent rearrangement or loss of ligand conformational flexibility). Obtained affinity for D-mannose is in millimolar range, which is in accordance with results reported previously for specific lectin–saccharide interactions.

Biological functions of lectins in corals are poorly understood. There are suggestions that they are important for symbiosis with algae and pathogen defense. Corals are endangered species and one of the reasons for their extinction is connected with the loss of symbiosis with algae. This study presents a novel type of lectin with unusual mannose specific structure and provides basis for understanding role of lectins in coral.

**SW06.S28–20**

**Effects of astragalus, lemon balm, clove, fenugreek and cinnamon on blood glucose level after oral glucose loading in rats**

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**Background and aim:** Herbs like astragalus, *lemon balm, clove*, fenugreek and cinnamon are consumed in various ways (dietary supplement, herbal tea etc.) all over the world. Consumption of these herbs (along with other health beneficial effects) has been mentioned to be helpful in decreasing blood glucose levels of dia-
Acetylation patterns of gangliosides in brain tissue of ganglioside-deficient mice

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Gangliosides are sialylated glycosphingolipids especially abundant in mammalian nervous system. With their carbohydrate moiety protruding from the cell, gangliosides interact with opposing cells and molecules of the extracellular matrix. One of the most common covalent modifications of gangliosides is O-acetylation of sialic acids which results in considerable changes in their physiological properties.

In order to investigate acetylation patterns of gangliosides we used genetically modified mouse models which lack ganglioside biosynthetic enzymes, St6gal1 null and B4galnt1 null. Total concentration of brain gangliosides was the same in mouse models and wild-type mice; however mice with disrupted St6gal1 gene lack b- and c-series while they accumulate a-series gangliosides. B4galnt1 null mice lack all complex gangliosides accumulating just GM3 and GD3. We performed structural analysis of accumulated ganglioside species with focus on acetylation pattern in brain tissue of ganglioside-deficient mice. The gangliosides were extracted and separated using high performance thin layer chromatography. Alkalai treatment was performed in parallel to determine the presence of O-acetylated ganglioside species. Separated ganglioside fractions were further analyzed by tandem mass spectrometry.

We found several O-acetylated ganglioside species in ganglioside-deficient mice not present in the wild-type mice. In B4galnt1 null mice we confirmed the presence of previously reported O-acetylated GD3, but also O-acetylated GM3 species was detected, while in St6gal1 null mice O-acetylated GD1a was found.

Although ganglioside-deficient mice have been used in research for years, structural composition of accumulated gangliosides has not been fully investigated. Since changed O-acetylation pattern could potentially contribute to altered ganglioside interactions with other molecules, this is a good model to explore the effects of acetylation on ganglioside function in crucial cellular events.
the precursor ion at \( m/z \) 225.6 and the fragment ions at \( m/z \) 74.7 (quantifier), 85.4 (qualifier) for the internal standard EtG-d5. A 6.00 min segmented analysis was performed. The LC-MS/MS method developed is cheap, repeatable, reproducible and an easy one, with acceptable measurement uncertainties. The sample preparation includes only dilution. Creatinine concentration was measured using spectrophotometric absorbance development of Jaffé reaction. The EtG measurement was linear between concentration range 100–3000 ng/ml. LOD and LOQ of the method was 104.21 and 165.00 ng/ml. Intermediate precision of the method was evaluated using ANOVA analysis and HorRat ratio. A cut-off limit determination study with 20 volunteers was performed and a cut-off limit of 817.63 ng/mg (concentration normalized to creatinine) was suggested for EtG positive-urine samples. We suggest that the real samples below this limit should not be legally regarded as having consumed alcohol. The concentrations of five real drunk samples were successfully predicted by using this method after zero HS-GC/MS blood alcohol concentration result. The measurement uncertainties of each sample result were calculated.

**Keywords:** Ethyl Glucuronide, LC MS MS, Determination in urine, Cut-off limit, Validation, Alcohol biomarker.

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**SW06.S28–24**

**GWAS of IgG glycome reveals importance of IgG glycosylation in a variety of diseases**

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Glycosylation of immunoglobulin G (IgG) influences IgG effector function by modulating binding to different Fc receptors. To identify genetic loci associated with IgG glycosylation, we quantitated N-linked IgG glycans in plasma of 2247 individuals from four European populations. After isolating IgG from human plasma, N-glycans were released and analyzed by ultra performance liquid chromatography (UPLC). Genome-wide association study (GWAS) identified 17 loci which associate with IgG glycome with genome-wide significance. Four loci contained genes encoding glycosyltransferases (ST6GAL1, B4GALT1, FUT8 and MGAT3), while the remaining loci that have not been previously implicated in protein glycosylation. However, most of them have been strongly associated with autoimmune and inflammatory conditions, including systemic lupus erythematosus, rheumatoid arthritis, ulcerative colitis, Crohn’s disease, diabetes type 1, multiple sclerosis, Graves’ disease, celiac disease, nodular sclerosis, and/or haematological cancers (acute lymphoblastic leukaemia, Hodgkin lymphoma, and multiple myeloma). This study revealed that IgG glycosylation is regulated by a complex network of genes through still unknown mechanisms. The observed pleiotropy with autoimmune diseases and haematological cancer indicates that individual variations in IgG glycosylation affect IgG function and contribute to disease development and progression.

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**SW06.S28–25**

**Heterogeneous expression and epigenetic regulation of d-glucuronyl C5-epimerase tumour suppressor gene in prostate cancer**

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Heparsulfate proteoglycans play an important role in cell-cell and cell-matrix interactions and signalling, and one of the key enzymes in heparansulfate biosynthesis is D-glucuronyl C5-epimerase (GLCE). A tumour suppressor function has been demonstrated for GLCE in breast and lung carcinogenesis; however, no data are available as to the expression and regulation of the gene in prostate cancer.

In this study, decreased GLCE expression was observed in 10% of benign prostate hyperplasia (BPH) tissues and 53% of prostate tumours, and increased GLCE mRNA levels were detected in 49% of BPH tissues and 21% of tumours. Immunohistochemical analysis revealed an intratumoural heterogeneity of GLCE protein levels both in BPH and prostate cancer cells, resulting in a mixed population of GLCE-expressing and non-expressing epithelial cells in vivo. A model experiment on normal (PNT2) and prostate cancer (LNCaP, PC3, DU145) cell lines in vitro showed a 1.5–2.5-fold difference in GLCE expression levels between the cancer cell lines and an overall decrease in GLCE expression in cancer expression. Methyl-specific PCR, bisulfite sequencing and deoxy-azacytidin (aza-dC) treatment identified differential GLCE promoter methylation (LNCaP 70–72%, PC3 32–35%, DU145 and PNT2 no methylation), which seems contribute to heterogeneous GLCE expression in prostate tumours. Ectopic GLCE expression in morphologically different prostate cancer LNCaP and PC3 cells resulted in differential changes in their expression profiles. According to the Cancer PathFinder RT Profiler PCR Array, most of the genes affected by ectopic re-expression of GLCE both in LNCaP and PC3 cells are involved in angiogenesis (ANGPT1, PDGFβ, JGF1, TEK, SERPINE1, TNF, ITGB3, ERBB2, IL8, MMP1, PLAUR) and invasion/metastasis (MMP1, MMP2, MMP9, SERPINE1, SERPINB5, PLAU, FAS, ERBB2, ITGB3) pathways, with cell line-dependent functional effects of GLCE expression.

The obtained results support the hypothesis on the involvement of GLCE in prostate carcinogenesis, reveal the complex deregulation of GLCE expression in prostatic diseases compared with normal prostate tissue and suggest that GLCE may be used as a potential model to study the functional role of intratumour cell heterogeneity in prostate cancer progression.

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**SW06.S28–26**

**TFEB is involved in the regulation of glycohydrolases lysosome-to-plasma membrane delivery**

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Transcription factor EB (TFEB) regulates multiple aspects of lysosomal dynamics, including the propensity of lysosomes to fuse with the plasma membrane (PM), suggesting that the range of bio-
logical functions of TFEB still need to be fully elucidated [1]. Currently many reports indicate the association of active lysosomal glycohydrolases with extra-lysosomal compartments. In particular, the presence of glycohydrolases on the PM has been demonstrated [2,3] and a possible role in the modification of the cell surface glycosphingolipids, participating to the modulation of cell functions such as cell-to-cell interactions and signal transduction pathways, has been proposed [4,5]. In this context, the coordinated expression of lysosomal glycohydrolases and their translocation to the PM could be crucial for many cellular events. In this work, we overexpressed TFEB in HeLa293 cell line and demonstrated that TFEB nuclear translocation drives to a significant increase of \( \beta \)-hexosaminidase (Hex) and \( \beta \)-galactosidase (Gal) activities on cell surface due to the activation of lysosome-to PM delivery. Glycohydrolases Hex and Gal are both involved in the stepwise degradation of GM1 to GM3 ganglioside. It is notable that monosialogangliosides are instrumental in the formation of PM lipid microdomains and participate to the modulation of signalling pathways by interacting with membrane proteins. Therefore, the association of Hex and Gal on the cell surface close their natural substrates and the modulation of their level by TFEB strongly suggest a role of these enzymes on the in situ PM remodelling to quickly respond to changes in cellular needs. In this contest, our data clearly indicate that TFEB is implicated on the regulation of lysosome-to-PM delivery of glycohydrolases, suggesting a possible role of TFEB on the translocation of lysosomal enzyme to the PM, not only in normal but also in pathological conditions.

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**References**


**SW06.S28–27**

**DAB derivatives as inhibitors of retaining glycosyltransferases**

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Glycosyltransferases (GTs) catalyse the transfer of monosaccharide moieties from activated nucleotide sugars to diverse glycosyl acceptors. These enzymes, which are responsible for the biosynthesis of oligo and polysaccharides and glycoconjugates, are classified as retaining or inverting depending on whether the stereochemistry of the anomeric carbon of the transferred sugar is the same or the opposite in the substrate and the reaction product. We have studied the inhibitory potency of several 1,4-dideoxy-1,4-imino-D-arabininitol (DAB) derivatives on two Leloir retaining GTs, glycosyl synthase from E. coli (EcGS) and isoform 4 of sucrose synthase from potato (SuSy 4), and one non-Leloir retaining GT, rabbit muscle glycogen phosphorylase (RMGP).

DAB is a known potent GP inhibitor, but there is scarce information about its ability to inhibit other GTs. In this work, 29 amino acid, amino alcohol and ring amino acid DAB derivatives were synthesised [1], following a published procedure, in order to study their relative inhibitory potency and their selectivity in front of three model retaining GTs. Although these enzymes possess a highly conserved catalytic site architecture, the DAB derivatives analysed, which were always found to be competitive inhibitors with respect to the glycosyl donor, showed extremely diverse inhibitory potential. Since GS and GP are the two key enzymes that control cellular glycolgen content, our approach may provide a tool to selectively inhibit one or the other enzyme and thus modulate glycoconjug metabolism.

**Reference**


**SW06.S28–28**

**Fluorescence anisotropy changes induced by aminoglycosides in artificial and natural membranes**

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Aminoglycosides are hydrophilic, polycationic, amine-containing carbohydrates that are binding both to the anionic outer bacterial membrane and to anionic phospholipids in the cell membrane of mammalian renal proximal tubular cells. Aminoglycoside antibiotics, largely used in infections with Gram-negative bacteria, are known to induce a pronounced nephrotoxicity and ototoxicity. The mechanisms by which these antibiotics interact with cell membrane are not yet fully understood. Our aim was to study the modification of membrane fluidity induced by these antibiotics. We tested the effect of 3 aminoglycosides: gentamicin, amikacin, and kanamicin on artificial (liposomes) and natural membranes (opossum kidney [OK] epithelia cells). Liposomes prepared from dimyristoyl-phosphatidylcholine (DMPC) mixed with cardiolipin, which mimick the heterogeneous charge composition of the natural cell membrane, were used. Membrane fluidity was assessed using fluorescence spectroscopy recordings on TMA-DPH labeled liposomes and OK cells. The most evident changes were observed in kanamycin treated cells and liposomes. At low temperature (31–33°C) a destabilization of lipid packing in membrane of OK cells was observed. The fluorescence anisotropy of liposomes was increased at all temperatures (15–35°C) in the presence of all antibiotics, especially kanamicin. A more pronounced membrane destabilization was observed in cardiolipin containing liposomes as compared to simple liposomes, suggesting that membrane charge is very important for adhesion of aminoglycosides. In conclusion both membrane models showed an altered membrane fluidity in the presence of the applied antibiotic.

**SW06.S28–29**

**Adhesins from Pichia pastoris – a structural basis for a symbiotic lifestyle?**

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Fungi and yeasts are able to colonize most known ecological niches. This enables them to adopt a broad variety of different lifestyles, from biofilms to floe formation. The basis for achieving different lifestyles is cell-cell communication as well as adhesion. In yeast, adhesion is accomplished by a set of GPI-anchored cell-wall proteins, where many of them belong to the fungal adhesin
superfamily with three distinct regions. By the A-domain carbohydrate residues in the cell walls of other cells or surfaces can be recognized in a lectin-like manner via a Ca\(^{2+}\)-ion. The highly repetitive B-region is thought to present the A-domain by its stalk-like shape on the outside of the fungal cell wall. The C-domain serves as fixation in the cell envelope via a GPI-anchor. On the basis of two previously known adhesion proteins, Epal from *Candida glabrata* [1], serving in adhesion to human epithelial cells, and Flo5, which mediates flo-c-formation in *Saccharomyces cerevisiae* [2], we now present fungal adhesion proteins from *Pichia pastoris*. We report structural, biochemical and cell biological results that indicate adhesion to chitin core-residues by one of these adhesins. This may be the first molecular clue for a social relationship between *Pichia* yeasts and insects, which are known for several strains from the *Pichia* genus.

References


SW06.S28–31

Polypotency of the immunomodulatory effect of pectins

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Pectins are the major component of plant cell walls and possess diverse biological activities including immunomodulatory properties. The pectin macromolecule contains fragments of linear and branched regions of polysaccharides as follows: homogalacturonan, rhamnogalacturonan-I, xylogalacturonan and apiogalacturonan. The aim of the present study is to investigate the relationship between the structure and immunomodulatory activity of pectins.

The backbones of pectic macromolecules possess immunosuppressive activity. Pectins containing >80% galacturonic acid residues were found to decrease macrophage activity and inhibit the delayed-type hypersensitivity reaction. Branched galacturonan fragments results in a biphasic immunomodulatory action. The branched region of pectins mediates both increased phagocytosis and antibody production. The fine structure of the galactan, arabinan and apiogalacturonan side chains determines the stimulating interaction between pectin and immune cells. The leukocyte system was shown the first to react after the administration of pectic polysaccharides. The multiple actions of pectins on immune reactivity may be caused by structural polypotency: pectins of the native plant cell wall appeared to possess capacity both to stimulation and to suppression of immune response. Immune resistance through the functional activity of leukocytes appears to depend on the ratio of the polysaccharide chains of galacturonan, rhamnogalacturonan, apiogalacturonan and other fragments extracted during the digestion of plant foods.

The data regarding the immunomodulatory effects of pectins that are orally administered likely reflect the evolutionary pattern of the immune response to the presence of pectic polysaccharides in the gastro-intestinal tract of mammals. For the integrated (holistic) picture of the organic world, mammals are suggested to use polypotential pectins by choosing plants for food and changing the properties of the gastro-intestinal medium. Moreover, the structural features of pectins provide information on the species composition of plants in the habitat of the animal, and this information is recognised by the immune system. Manipulation of composition of pectic fragments in the human gastro-intestinal tract is supposed as a new approach of immunomodulatory therapy.
genes; the universal genome with genes common for all strains; and the shared periphery (genes present in a subset of strains).

We performed pairwise comparing of pan-genomes of E. coli, Shigella spp. and S. enterica. The species-specific, universal genes of dual pan-genome reflect specialization of the group and may be used to delineate fine taxonomic relationships within a larger taxonomic category. The gene complements of E. coli and Shigella spp. are very similar and these species cannot be separated into two groups, as they have a common gene pool.

At the same time, despite intensive HGT between Enterobacteriaceae species and specifically between E. coli and S. enterica, the latter maintain stable species restricted gene pools.

GO analysis was performed to identify functional categories linked to different parts of combined pan-genome. For instance, the S. enterica-specific genes group contains genes of cobalamin biosynthesis, the E. coli-specific group contains transporters, and the ‘pathogenesis’ category is overrepresented in several pan-genome parts.

Genes that are shared between some, but not all, strains normally form two categories, dependent on the fraction of strains carrying a gene. The genes of the shared periphery may have different evolutionary histories, being inherited vertically from the ancestral pan-genome (mainly genes from the frequent periphery) or transferred horizontally (mainly rare periphery). These groups differ also in functional characteristics, being enriched in different functional classes, both metabolic and structural.

**SW06.W29–2**

Using biological networks to understand mycobacterial pathogens

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Mycobacterial species are responsible for a number of important human diseases, most notably tuberculosis and leprosy. The genome sequences of a number of mycobacterial pathogens and non-pathogens are now available for exploitation. In our work we use comparative genomics and data integration to do in silico analysis of microbial pathogens at the systems level to better understand the molecular biology of the organisms. We have generated functional interaction networks for three mycobacterial species as well as a TB-human host-pathogen interaction network and used these to study the evolution of organisms and their interaction with the host system. We are also using networks for the downstream analysis of genome-wide association studies and have applied these to study susceptibility to tuberculosis in an admixed population. Several aspects of this work will be presented, with a focus on Mycobacterium tuberculosis, the causative agent of tuberculosis.

**SW06.W29–3**

Characterization of newly isolated lytic *Thermus thermophilus* bacteriophage phiFa

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**Background:** Bacteriophages are the most abundant and the most diverse form of life on our planet. However phages that infect thermophilic eubacteria have remained mostly unexplored. More than one hundred of bacteriophages that infect *Thermus* have been isolated, and only few of such phages were completely sequenced and investigated in terms of their morphology, genomics and transcriptional regulation. Here we report a preliminary characterization of a newly isolated lytic bacteriophage of hyperthermophilic eubacterium *Thermus thermophilus* HB8. The bacteriophage phiFa was isolated from the hot mud of Ischia island (Naples, Italy) in October 2013. To our knowledge, it is the first bacteriophage of *Thermus thermophilus* isolated from the Vesuvio region.

**Objectives:** Sequence and bioinformatics analysis, and characterization of novel bacteriophage.

**Methods:** Electron microscopy, total DNA purification, high throughput sequencing of phage genomic DNA, genome assembly and annotation techniques were utilized to characterize the bacteriophage phiFa.

**Conclusion:** Electron microscopic examination indicated that phiFa is an unusual tail-less bacteriophage with anicosahedral head, which most likely belongs to a Podoviridae family. Analysis of the DNA restriction digest patterns and partial sequencing indicate that phiFa is not related to any known *Thermus* phages. The genome of phiFa was completely sequenced. It is linear and consists of 71 kb and 79 putative ORFs. The G+C content (59.2%) is significantly lower than that of the host, *T. thermophilus* (69.4%). Similarly to other bacteriophage genomes, the coding density of the phiFa genome was high. Only 30% of ORFs displayed limited sequence homology to the corresponding genes of *T. thermophilus* bacteriophages P23-45, P74-26, YS40, and IN93, which belong to a different morphology group of *Thermus* phages. The details of phage genomics and regulation will be discussed.

**SW06.W29–4**

In silico characteristics of structure and activity of the food-derived peptides with an ACE inhibitory bioactivity

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It is scientifically evidenced that cardiovascular diseases including hypertension are directly related to diet. The nutritionists claim that peptides reducing blood pressure found in food are more natural and safe medications than traditional antihypertensive drugs. So far, ACE inhibitors (peptides responsible for vasodilation) derived from food proteins have been found as the best known group of biologically active peptides. Their ‘natural’ origin (food) as well as biofunctionality make them as the promising alternative when making choice between the synthetic drugs and naturally originated bioactive food [1].

Identification of specific molecular properties which decide about peptide biological activity can be an attribute considered in production of nutraceuticals and functional food. Laboratory experiments as well as chemometrics modelling techniques are involved to discover the impact of the structure of ACE (angiotensin converting enzyme) inhibitors on their bioactivity. Chemo-metrics techniques applied in contemporary life sciences are strongly intertwined with chemoinformatics [2].

The aim of the study was to apply chemoinformatics techniques to elucidate the relationships between the triple amino acid sequences of ACE inhibitors and their activity expressed by the concentration corresponding to their half-maximal activity (IC50). The population of over one hundred peptides retrieved from the database of bioactive sequences (BIOPEP: www.uwm.edu.pl/biochemia) [3]. Molecular descriptors (including the ones derived from quantum chemical calculations), defined the structural properties of amino acids being the components of the ACE inhibitors, originated from CODESSA [4] program used for the QSAR analysis.
For some of the peptides, their experimental vs. predicted activities were concurrent. Such peptides were similar in terms of their chemical nature – they consisted of C-terminal proline, the crucial amino acid in the inhibition of angiotensin converting enzyme (ACE). Our findings were consistent with results of the scientists analyzing the relationship between structure and activity of ACE inhibitors by means of other in silico methods [5].

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References

SW06.W29–5
Hydrophobic segment: an essential domain for cytotoxicity of dimeric ribonucleases?
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The cytotoxic ribonucleases (RNases) are found among different organisms. Over the past decade there has been increasing research in the RNase functions related to the gene expression control, cell growth and differentiation, cell protection from pathogens, and apoptosis induction. RNases with special biological actions include bovine seminal RNAse (BS-RNase) and RNAse from Bacillus intermedius 7P (binase), both endowed with a strong antitumor effect and capable to the dimerization [1,2]. Although the number of resolved RNase three-dimensional structures rapidly increases, little has been published on the structural key of the cytotoxicity.

The exact mechanism by which cytotoxic RNases reach the cytosol of tumor cells remains unclear. The interaction of RNases with a lipid bilayer is involved in the translocation of RNase molecule across the endosomal membrane [3]. The main aim of this investigation is to study the hydropathy character of toxic antitumor RNases (bovine seminal RNAse and binase) and two non-toxic RNases (bovine pancreatic RNAse, or BP-RNase, and human pancreatic RNAse, or HP-RNase) by sliding-window hydrophobicity analysis. Comparative hydropathy plot analysis of the non-toxic pancreatic RNases and their toxic BS-like variants, called BBP-RNase [4,5] and HHP-RNase [6], was also performed. The data obtained indicate that all above-mentioned cytotoxic RNases have a hydrophobic (HΦ) segment, which is sterically available for the hydrophobic interaction with a tumor cell membrane and endosomal membrane. Remarkably the HΦ segment is not identified in the amino acid sequences of non-toxic RNases. It was shown that the HΦ segment of BS-RNase, binase, BBP-RNase, and HHP-RNase is located mostly in helix II.

Innovative results were generated using the helical wheel representation of the helix II of BS-RNase and binase. Transfer free energy and hydrophobic moment values as a function of α-helical content was calculated. The approach provides a reasonable explanation on a key role for the leucine residues of helix II in the cytotoxicity mechanism of the RNases. From this it is necessary to conclude that a HΦ segment may have a double function for both the dimerization and the hydrophobic interaction of the cytotoxic RNases with a lipid bilayer.

In contrast to the native helix II of non-toxic pancreatic RNases, the HΦ segment of BBP-RNase and HHP-RNase demonstrates an extremely high thermodynamic favorability to the interfacial region of a lipid bilayer. Although the technique of hydropathy plots allows to identify the regions of polypeptide with hydrophobic properties, the three-dimensional structure of these regions is important to investigate. Localization of the HΦ segment of cytotoxic RNases was identified using a molecular modeling approach. It was obtained that hydrophobic amino acid residues of the HΦ segment of BS-RNase and binase are exposed on surface of the monomeric molecules. It is mean that the HΦ segment is sterically accessible for hydrophobic interaction both with a lipid bilayer and another RNase molecule during the dimerization.

RNases are water-soluble globular proteins. However, they can interact with a lipid bilayer and the interaction is thermodynamically favorable. After cytosolic internalization the RNases can interact with organelle membranes. The best candidate for this role is a mitochondrial outer membrane. It is known that the mitochondrial outer membrane permeabilization (MOMP) is crucial factor for the induction of apoptosis [7]. The main idea of our perspective research is to show that the toxic RNases with a HΦ segment can promote the apoptosis of tumor cells by engaging the mitochondrial pathway. It may well be that the toxic RNases with a HΦ segment can disturb a tumor cell’s rest in the same manner as multidomain Bcl-2 proteins Bax and Bak when MOMP occurs.

It was discovered that some cytotoxic RNases contain a HΦ segment located in helix II, which is capable of thermodynamically favorable interaction with a lipid bilayer. It was shown that Leu-32 can play a key role for the dimerization of binase. The new theory of the cytosolic internalization of toxic RNases comprising a HΦ segment was proposed.

References
LigandRNA: computational predictor of RNA-ligand interactions
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Recently nucleic acid molecules have become an attractive drug target due to the increasing awareness of their importance in key biological processes. The functions of many RNAs are dependent on their interactions with other molecules, including proteins, other nucleic acids, and small molecules. In particular, many regulatory RNAs exert their function by interacting with small molecule ligands. For example, the majority of antibiotics (e.g. aminoglycosides, macrolides) target the ribosome, and in particular active sites composed of ribosomal RNAs. The other drug-attractive RNA class are riboswitches that are mRNA-embedded elements which can directly bind a ligand, and in this way regulate the gene function without the necessity of protein co-factors. The rapid increase in the number of solved RNA 3D structures allowed for a structure-based approach to the discovery of new ligands acting on RNA. However, the RNA-ligand docking problem still remains awkward, especially when compared to the progress made in the development of an analogous protein-ligand docking methods.

This motivated us to create the LigandRNA, a software-independent RNA-ligand specific scoring function that is able to rank and validate RNA-ligand complexes regardless of the procedure used to generate them. Our method employs a grid-based algorithm and a knowledge-based potential derived from ligand binding sites in experimentally solved RNA-ligand complexes. LigandRNA can be used in a fully predictive mode to identify or to design drugs targeting RNA (e.g. novel antibiotics targeting bacterial ribosomes or novel inhibitors affecting riboswitches structure and function), or it can be used to support third-party docking programs by scoring ligand poses generated by them. Moreover, in order to increase prediction accuracy we decided to merge the LigandRNA knowledge-based and Dock6 force field approaches to create a combined scoring function. The consensus scoring function results in the significant increase in tracking near-native ligand poses. The results show that the LigandRNA scoring function separately selects binding geometries with RMSD < 2 Å in 43% of all cases and the combination of LigandRNA and Dock6 gives 62% correctly predicted ligand poses. The LigandRNA program is available free of charge as a web server at http://ligandrna.genesilico.pl.

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Molecular systematics and evolution of A. mystacinus (Mammalia:Rodentia) inferred from cytb gene sequences
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Apodemus mystacinus is a Rodent species distributed in Asia Minor, Aegean Islands and Syria. Although this species had five subspecies, one of this subspecies has been promoted to species level and called A. epinolas. In this study mtDNA cytb gene sequences were used to infer phylogeny and the time of diversification of A. mystacinus populations. Our results suggested that three different lineages of A. mystacinus distributed in Asia Minor, Crete and Syria as A. m. eueinus, A. m. mystacinus and A. m. symnensis. These populations were differentiated in Pleistocene (approximately 1.3 Mya) according to evolutionary diversification sums. Considering the latitudinal and longitudinal spread of populations, the topographic structure of Anatolia may be offered suitable micro refuges leading the differentiation of species with the effect of the climatic fluctuations in Pleistocene.
structed two hypothesis of TP distribution on set of coding sequences and simulated corresponding artificial datasets. We explored modeling sets of two types: the first one where all sequences TP were obtained from one TP pattern (Perf) and the second one where conversely TP of all sequences were independent and random (Rand). We found that triplet periodicity more similar inside genome than between genomes and that TP distribution inside genome corresponds to hypothesis which imply common TP pattern for majority of sequences inside a genome (Perf). Additionally we performed gene classification based on triplet periodicity matrices. This classification showed that triplet periodicity allows to identify genome to which a given gene belongs with more than 85% accuracy for the most cases. Our results suggest that there is some process inside genomes that formed and maintained special TP type of genes inside one genome. Without such process it is hard to explain how TP could persist in the context of mutation process even if all genes inside genome initially had the same TP type. In practice genome-specificity of TP could be useful for pathological genome identification in medicine and homogeneity of TP inside genome – for prediction of horizontally transferred genes.

**SW06.W29–11**

**Molecular dynamics simulation approach for DNA duplex thermal stability prediction**

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Due to significant progress in development of computer software and hardware the *in silico* research became a useful tool to study biopolymers properties. Development of derivatives and analogues of nucleic acids remain laborious, costly and time-consuming. The using of computer simulations may allow precalculate physico-chemical properties of new derivatives before chemical synthesis. The aim of this work is to study a molecular dynamics (MD) simulation approach for nucleic acid thermal stability calculation.

Using Amber 11 software (UCSF, USA) we simulated single and double stranded oligodeoxynucleotides. The enthalpies of DNA duplex formation were calculated as a difference of the total internal energy of double- and single-stranded states which were averaged from 10 ns MD trajectory. Computations were performed on NVIDIA GTX580/Intel i7-2600 hardware and resources of Siberian supercomputer center (ICMMG SB RAS). The use of GPU has speeded up the modeling in implicit solvent up to 60 times and up to 30 times in explicit solvent in comparison with the one node of CPU.

To determine optimal parameter set of modeling we have used Dickerson-Drew dodecamer (DDD) 5’-CGCGAATTCGCG-3’ with well characterized secondary structure and thermal stability. We have varied force field, temperature, heating protocol, and ion concentration in implicit and explicit solvent, solvent shell radius and compared averaged double stranded DNA structures with those experimentally obtained. We have determined the optimal parameters of modeling in implicit and explicit solvent. It was shown that the experimental and obtained via the MD simulation conformations of duplexes structures are close to each other. Also the difference of experimental and calculated via the MD simulations enthalpies differ <15% whereas the experimental accuracy is about 10%.

To verify the MD predictive ability we have collected database of experimentally determined thermodynamic parameters (enthalpy and entropy) of hybridization of more than 300 oligodeoxynucleotides. The length of oligonucleotides varies from 4 up to 16 base pair (avver. 9 bp), GC-content 0–100% (avver. 57%). The total energy of oligonucleotide or duplex was averaged over 10 000 snapshots of 10 ns trajectories simulated with optimal parameter set. We have observed high correlation between the values of hybridization enthalpies obtained experimentally and calculated using MD in implicit and explicit solvent. The best prediction of thermodynamic parameters was obtained in explicit solvent after analysis of 10 ns MD trajectories using Molecular Mechanics Poisson Boltzmann Surface Area (MMPBSA) calculations at 300K.

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good approaching to the widely used empirical nearest neighbor model allows to predict internal energies of DNA duplex formation and melting temperatures with accuracies 10% and 1.5 °C, respectively [1].

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Reference


**SW06.W29–12**

Bioinformatic analysis of family GH101 of glycoside hydrolases

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Recent progress in genome sequencing resulted in a rapid growth of the sequence databases. The number of known representatives of all protein families has increased dramatically. It naturally raises a question if the existing protein classifications are still stable or appearance of ‘the intermediate forms’ washed out the clear borders between protein families or subfamilies. As a case study, we choose family GH101 of glycoside hydrolases.

On the basis of sequence similarity of the catalytic domains all glycoside hydrolases have been grouped into more than 100 families (GH1-GH132). Family GH101 includes retaining endo-α-N-acetylglactosaminidases (EC 3.2.1.97) and their uncharacterized homologues (totally 100 proteins) [1]. This family was described in 2005 [2]. Four years ago we revealed 95 non-identiﬁed homologues (totally 100 proteins) [1]. This family was described in 2005 [2]. Four years ago we revealed 95 non-identiﬁed homologues (totally 100 proteins) [1]. This family was described in 2005 [2]. Four years ago we revealed 95 non-identiﬁed homologues (totally 100 proteins) [1]. This family was described in 2005 [2]. Four years ago we revealed 95 non-identiﬁed homologues (totally 100 proteins) [1]. This family was described in 2005 [2]. Four years ago we revealed 95 non-identiﬁed homologues (totally 100 proteins) [1].

Iterative screening of the database allowed us to reveal 15 090 proteins homologous to GH101 domain. This family is still quite distinct and its closest neighbor is GH129 family of Actinobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia. Pairwise sequence alignment and phylogenetic analysis allowed us to distinguish six subfamilies (101a-101f) in the GH101 family. Iterative screening of the protein database by PSI-BLAST revealed the closest relationship of GH101 domains with GH129 (or GH1) domains. More distant similarity was found with some proteins from GH13, GH20, GH27, GH29, GH31, GH36, GH66, GH97, COG1306, and COG1649 families [3–5].

Recently we did another analysis of the GenPept database. Iterative screening of the database allowed us to reveal 15 090 proteins homologous to GH101 domains. They represent several families of glycoside hydrolases having the TIM-barrel type catalytic domains. Particularly we found 345 proteins containing the GH101 domain. This family is still quite distinct and its closest neighbor is GH129 family of α-N-acetylglactosaminidases (EC 3.2.1.97). In order to additionally increase the number of proteins for phylogenetic analysis we also used 17 GH101-containing proteins found in the Integrated Microbial Genomes database [6]. All obtained proteins belong to the same four bacterial phyla as 4 years ago with clear domination of representatives from Actinobacteria and Firmicutes. We found no GH101-containing proteins from Proteobacteria despite the fact that almost a half of all bacterial genome projects corresponds to this phylum [7]. According to the phylogenetic analysis, the subfamily structure retains stable: almost all proteins can be easily classiﬁed in six subfamilies described by us 4 years ago [3]. All subfamilies compose stable clusters on the tree. In contrast to many other glycoside hydrolase families, the role of lateral gene transfers and gene duplications was minor during the evolution of genes encoding GH101-containing proteins.

References


**SW06.W29–13**

Structural analysis of cytochromes P450 for development of new molecular dynamics force field parametrization

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More than 10 000 isoforms of cytochrome P450 have been identiﬁed to date [1], mainly by DNA sequence analysis. Many studies are focused on cytochrome P450 due to its key role in sterol metabolism and xenobiotics conversion in humans. Computer simulations are used extensively in studies related to the rational drug design, mostly relying on molecular dynamics (MD) simulation.

Heme moiety of cytochrome P450 is bound to cysteine as a proximal ligand. First strategy for deriving of harmonic parameters conﬁrming Amber formulation of force ﬁeld included statistical analysis of all crystallographic structures stored in PDB and CSD. Obtained distribution for bond lengths and angles were used to calculate corresponding force constants as described by David et al. [2]. It was found that distribution for length of Fe-S bond (1294 values accounted) has two peaks, major one at 2.32 and minor at 2.85 Å. Latter is supposed to be an evidence of broken bond between iron and sulfur atoms.

Another approach used is quantum chemistry calculations. We chose simpliﬁed structure of porphin with embedded iron atom having thiometyl group at 5th ligand position to represent key properties of heme. Force constants were derived by means of semi-numerical hessian calculations with UHF method for multiplicity 6 followed by DFT calculations with B3LYP functional at different extent of atoms’ displacement.

Our results provide the basis for studies involving calculations of long molecular dynamics trajectories of cytochrome P450 and ﬂexible docking of compounds into an active site accounting normal modes of the enzyme. We have developed new methods for determination of binding energies using molecular dynamics simulation, as well as spectroscopic approaches to study the molecular interaction between potential antifungal compounds and their molecular targets.

Reference


Evaluation method for the potential functionome harbored in the genome and metagenome

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One of the main goals of genomic analysis is to elucidate the comprehensive functions (functionome) in individual organisms or a whole community in various environments. However, a standard evaluation method for discerning the functional potentials harbored within the genome or metagenome has not yet been established. We have developed a new evaluation method for the potential functionome, based on the completion ratio of Kyoto Encyclopedia of Genes and Genomes (KEGG) functional modules.

Distribution of the completion ratio of the KEGG functional modules in 768 prokaryotic species varied greatly with the kind of module, and all modules primarily fell into four patterns (universal, restricted, diversified and non-prokaryotic modules), indicating the universal and unique nature of each module, and also the versatility of the KEGG Orthology (KO) identifiers mapped to each one. The module completion ratio in eight phenotypically different bacilli revealed that some modules were shared only in phenotypically similar species. Metagenomes of human gut microbiomes from 13 healthy individuals previously determined by the Sanger method were analyzed based on the module completion ratio. Results led to new discoveries in the nutritional preferences of gut microbes, believed to be one of the mutualistic representations of gut microbiomes to avoid nutritional competition with the host.

The method developed in this study could characterize the functionome harbored in genomes and metagenomes. As this method also provided taxonomical information from KEGG modules as well as the gene hosts constructing the modules, interpretation of completion profiles was simplified and we could identify the complementarity between biochemical functions in human hosts and the nutritional preferences in human gut microbiomes. Thus, our method has the potential to be a powerful tool for comparative functional analysis in genomics and metagenomics, able to target unknown environments containing various uncultivable microbes within unidentified phyla.

Association between polymorphism of MTHFR c.677C>T and risk of cardiovascular disease in Turkish population: a meta-analysis for 2.780 cases and 3.022 controls

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Objective: Cardiovascular disease (CVD) remains the main cause of morbidity and mortality around the world. A common polymorphism c.677C>T has been identified in the gene coding for methylenetetrahydrofolate reductase (MTHFR), which is involved in the remethylation of homocysteine, and may predispose to CVD. A meta-analysis was performed to estimate the risk of CVD associated with MTHFR c.677C>T in Turkish population.

Method: Published studies were retrieved from PubMed, Science Citation Index/Expanded, Google Scholar, Turkish Medline, and the Turkish Council of Higher Education Theses Database. For each study, we calculated odds ratios and 95% confidence intervals, assuming frequency of allele and homozygote comparison, dominant and recessive genetic models.

Results: Thirty-one separate studies were included and 2.780 cases/3.022 controls were involved in the current meta-analysis. Significant association was found between c.677C>T polymorphism and risk of CVD when all studies pooled with random-effects model for T vs. C (OR = 1.33; 95%CI = 1.11–1.59; p = 0.002), TT vs. CC (OR = 1.87; 95%CI = 1.35–2.60; p < 0.001), TT+CT vs. CC (OR = 1.32; 95%CI = 1.06–1.64; p = 0.014) and TT vs. CT + CC (OR = 1.75; 95%CI = 1.29–2.57; p < 0.001). Further analysis indicated the significant association between MTHFR TT genotype and groups with venous thrombosis, peripheral arterial thrombosis, acute MI /MI. No publication bias was observed in any comparison model.

Conclusion: In conclusion, meta-analysis has an important role in medical research, public policy, and clinical practice. Our results support an association between the MTHFR c.677C>T polymorphism and the risk of CVD in Turkish population. This finding may potentially be important when considering pharmacogenetic cardiovascular therapies.

Molecular dynamics simulation of negatively charged DPPC/DPPI lipid bilayers and their interactions with I-BAR domains

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Newly discovered I-BAR domain proteins modulate the shape of negatively charged membranes allowing formation of filopodia. To date little is known about the specific interactions of these proteins with membrane. Molecular dynamics simulation is a powerful method, which allows to model the membrane-protein interactions. In the present study we investigated dipalmitoylphosphatidylcholine (DPPC)/dipalmitoylphosphatidylinositol-4,5-bisphosphate (DPPI) lipid bilayers in two levels of resolution: united-atom and coarse-grained. All simulations were carried out in Gromacs 4.5.3.

First, we investigated a bilayer composed of 127 DPPC and 1 DPPI using a united-atom force field Gromos53a6 and compared it to a bilayer containing larger number of DPPI (12). Both systems were simulated for 100 ns. We compared several parameters: area per lipid, volume per lipid, bilayer thickness, diffusion coefficient and deuterium order parameter (all of them were averaged over the simulation time). All these parameters were close to experimentally obtained parameters of the pure DPPC membrane. For the bilayer with larger number of DPPI we observed reduction of most parameters, except the bilayer thickness and deuterium order parameter, which were higher.

Next, using a coarse-grained force field Martini 2.2P we investigated a larger system: a bilayer composed of 1160 DPPC and 120 DPPI. To characterize the lipid bilayer at new resolution level we analyzed the same set of parameters. The most remarkable difference was observed in the value of the diffusion coefficient ~12*10^-7 cm^2/s which was almost ten times higher than for united-atom simulation. This acceleration of dynamics in coarse-grained models must be considered for proper evaluation of system properties.

Finally, we applied the I-BAR domains of MIM and Pinkbar proteins on top of the model bilayer and performed coarse-grained simulations for both systems. Presence of the proteins leads to changes in charged lipids distribution within the membrane.
Our data indicate that negatively charged DPPi lipids promote ordering of DPPC lipids leading to the formation of micelles within the membrane. The presence of the I-BAR domains stabilizes these conditions.

**SW06.W29–17**

In silico evaluation of the integration of *Agrobacterium* VirE2 protein into a lipid membrane

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Soil bacteria of the genus *Agrobacterium* are a natural vector for the transfer of genetic information (T-DNA) into plant and animal cells. It is believed that *Agrobacterium* VirE2 protein forms a membrane-spanning pore for the promotion of translocation of ssDNA across the membrane [1]. The aim of this work was to perform a computer evaluation of the integration of VirE2 protein into a lipid membrane. For the formation of a complex consisting of two and four VirE2 proteins, we used the program GRAMM-X (http://vakser.bioinformatics.ku.edu). For the integration of VirE2 protein into a lipid membrane, we used the CHARMM-GUI-Membrane Builder program (http://www.charmm-gui.org). We used the Membrane Builder, we integrated model structures consisting of one, two, and four VirE2 proteins into the membrane. The MOLE program (http://mole.chemi.muni.cz) was used for evaluation of the inner holes in VirE2, and up to three channels with diameters of 0.2–0.8 nm were observed. In a model structure formed from two and four VirE2 molecules in the membrane, we showed the formation of pores with channel diameters of 1.2–1.6 and 1.4–4.6 nm, respectively. Using the program MDWeb (http://mmb.irbbarcelona.org/MDWeb), we applied an MD simulation of VirE2 protein incorporated into the lipid membrane. According to the preliminary MD evaluation of the VirE2-membrane complex, we observed gaps in the PDB VirE2 protein structure in the following areas: GLN345, ALA439-PHE447, LEU472-SER476 that cause the unstable the MD simulations.

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**References**


**SW06.W29–18**

Sequence analysis around transmembrane regions and discrimination of subcellular localization of type II membrane proteins

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In order to carrying out protein functions, biosynthesized proteins should be transported to particular organelles. Signal-peptides, which are responsible for transporting proteins to the Endoplasmic Reticulum, and nuclear localization signals, which transport proteins to the nucleus, contain information about the subcellular localization of proteins and are located in amino acid sequences. The mechanism of signal sequences in soluble proteins have been clarified by many experimental approaches, however membrane proteins are thought to be localized by different process from soluble proteins. On the other hand, the lipid bilayers of each organelle consist of different kinds and ratios of lipid molecules, and have individual characteristics. Therefore, their transport signals are thought to recognize conformation and physicochemical properties of each membrane, and the characteristics of the signals are recognized by each organelle.

To find the characteristics of the transport signals that are localized to organellar membranes including the Endoplasmic Reticulum, the Golgi apparatus, and the plasma membrane from their amino acid sequences, the sequences around the hydrophobic regions of membrane proteins were analyzed, and a computational discrimination method was developed in this study.

Data of type II membrane proteins, singlepass type membrane localized proteins which have one hydrophobic region, were extracted from Uniprot Knowledge Base/SwissProt Release 2011_11. Hydrophathy profiles of each protein were estimated by average hydrophathy calculation. As a result, the most hydrophobic positions in each protein within the 100 amino acid residues from the N-terminus were included in annotation regions as transmembrane helices. The sequences were aligned at the most hydrophobic positions. Hydrophathy profiles and position specific amino acid propensities were different according to sequences of each organelle in N-terminus side than these hydrophobic regions. Therefore, it is thought to be possible to predict subcellular localizations of membrane proteins through the characteristic and optimization of hydrophathy profiles and amino acid compositions of hydrophobic regions around N-terminus side of each organelle dataset.

**SW06.W29–19**

Activation of NADPH oxidase subunit NCF4 (p40phox) induces ROS-mediated EMT signaling in HeLa cells

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The epithelial–mesenchymal transition (EMT) is a critical biological process characterized by morphological and behavioral changes in cells. The regulatory and signaling mechanisms of both developmental and pathological EMT have been investigated. Reactive oxygen species (ROS) play a role in early EMT, but the exact mechanism by which ROS are involved is unclear.

We investigated ROS-mediated EMT in human HeLa cells. Transforming growth factor beta (TGF-β) treatments lead to dramatic NADPH oxidase 2 (NOX2) inductions in HeLa cells; antioxidant treatment prevented TGF-β-driven EMT. Over-expression of the p40phox subunit (NCF4) led to activation of the NOX2 complex and ROS production. We showed that NOX2 and NOX5 mRNA was increased, along with increased expression of several matrix metalloproteinases (MMPs) in response to NCF4 expression. Moreover, these changes were reversible upon ROS scavenging.

Down-regulation of E-cadherin and up-regulation of Snail, Slug and vimentin occurred at the transcriptional level. We also showed that new EMT regulator, YB-1 is a downstream target in ROS-induced EMT. Together, these data suggest that ROS switching is necessary for increased EMT but is not required for the morphological changes that accompany EMT.

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SW06.W29–20
MisPred: quality control of gene predictions and public databases
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Despite significant improvements in computational annotation of genomes, sequences of abnormal, incomplete or incorrectly predicted genes and proteins remain abundant in public databases. Since the majority of these erroneous entries are not annotated as such, these errors seriously affect the reliability of these databases.

The main objective of the MisPred project is to develop tools to identify erroneous (mispredicted, abnormal or incomplete) sequences primarily from eukaryotic genomes in order to improve the quality of predictions and to maintain a database for erroneous proteins in order to inform the users on the quality of predictions.

Here we show that the MisPred computational pipeline may provide an efficient means for the quality control of gene predictions and databases. The rationale of the MisPred approach is that a sequence is likely to be incorrect if some of its features conflict with our current knowledge about protein-coding genes and proteins. The current version of MisPred uses eleven types of protocols to identify different types of sequence errors.

Analysis of the UniProtKB/Swiss-Prot, UniProtKB/TrEMBL, NCBI/RefSeq and EnsEMBL databases has revealed that – with the exception of Swiss-Prot – the proportion of erroneous sequences is very high in these databases.

MisPred works efficiently in identifying erroneous protein sequences and it also guides the correction of mispredicted sequences. We suggest that application of the MisPred approach will significantly improve the quality of gene predictions and the associated databases.

SW06.W29–21
New bioinformatics tools for RNA 2D/3D structure prediction, modeling, and analysis
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In analogy to proteins, the function of non-coding RNA molecules depends on their structure and dynamics, which are encoded in their linear sequences. Understanding of sequence-structure-function relationships provides an opportunity to predict structures and functions from sequences. This has proven to be a difficult task, however a few practically useful strategies have been identified for protein structure prediction. We developed methods based on a conjecture that essentially the same principles as those used to model protein structures are applicable to model RNAs – despite many obvious differences between these classes of macromolecules. As a proof of principle, we present our recently developed tools for RNA 3D modeling and analysis based on algorithms successfully used for protein structure prediction. We have developed software for RNA 2D structure meta-prediction, 3D structure prediction by comparative and de novo folding approaches, for RNA-protein docking, prediction of RNA-metal ion interactions, prediction of RNA 3D model accuracy, analysis of X-ray crystallographic data for RNA, multiscale modeling with experimental restraints and others. We have also developed programs for the analysis of interactions in RNAs and RNA-protein complexes. Our methods are available at http://genesilico.pl

Highlights of our RNA bioinformatics toolkit include: Mod-eRNA is a comparative modeling method. It requires a 3D structure of a template RNA molecule, and a sequence alignment between the target to be modeled and the template. It can model posttranscriptional modifications. ModeRNA can model the structures of RNAs of essentially any length, provided a homologous template structure exists. Recently, we have extended this method to use multiple templates, which improves modeling in conserved as well as variable regions.

SimRNA can fold RNA 3D structure starting from sequence alone. It is based on a reduced representation of the polynucleotide chains, uses a Monte Carlo sampling scheme, and a statistical potential to estimate the free energy. The scoring function reproduces a characteristic funnel-like shape for energy vs model quality. SimRNA is capable of finding a native-like conformation for RNAs <100 nt without any restraints, and much longer molecules if additional data are available such as secondary structure and long-range tertiary contacts.

CompaRNA is an online system for continuous evaluation of RNA structure prediction methods, which allows for objective testing and head-to-head comparison of web servers and stand-alone programs for prediction of secondary and tertiary RNA structure. Structures of RNAs deposited in PDB and those in the RNASTRAND database are used as the benchmark, revealing strengths and shortcomings of different algorithms.

SW06.W29–22
Evolutionary decline for a nuclear-encoded human mitochondrial aminoacyl-tRNA synthetase
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Mammalian mitochondrial (mt) aminoacyl-tRNA synthetases are nuclear-encoded essential actors for translation. Due to an endosymbiotic origin of the mitochondria, many of them are of bacterial type and share structural domains with homologous bacterial enzymes of same specificity. This is also the case for human mt aspartyl-tRNA synthetase (AspRS), which shares the bacterial insertion domain (BID) with bacterial AspRSs. The present study investigated an alternative spliced transcript of mt-AspRS mRNA. This isoform lacks exon 13 that codes for a region of the BID. The alternative transcript was present in all tested tissues; co-existed with the full-length form, possesses 5′-and
3’-UTRs, a poly-A tail and was bound to polysomes, indicating that it was actively translated. The corresponding protein was difficult to express in vitro and not detectable in cellulo or in vivo, strongly suggesting decreased protein stability. Bioinformatic analyses revealed divergence of the BID sequences in opisthokonts and protists that distinguished them from the bacterial and viridiplantae sequences, pointing to a loss of evolutionary pressure on this domain in non-viridiplantae AspRSs of mitochondrial location. The relaxed selective pressure combined with the occurrence of alternative splicing, involving a single structural sub-domain of likely no function, suggests that the new isoform serves as evolutionary playground towards possible reshaping of this domain. This evolutionary divergence of the BID is in line with other characteristics, established for the human mt-AspRS, that indicate functional relaxation of non-viridiplantae mt-AspRS when compared to the bacterial and plant ones, despite their common ancestry.

SW06.W29–23
Exact solution to protein alignment
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Most solutions to protein alignment problem have been found using available computer programs, such as BLAST, which is currently one of the most widely used. Computer programs tend to use various approximations, such as various penalties for deletions, insertions and gaps, they may use empirical parameters, statistics and ‘trial-and-error’ searching for pairing of amino acids. For some 40 years and more exact solution of protein alignment problem was for most part considered at best elusive, or even not possible. Few years ago we introduced a 20 × 20 amino acid adjacency matrix (AAA), which records presence of pairs of adjacent amino acids. On one hand similar proteins are expected to have similar amino acids and on the other hand matrix invariants (mathematical properties of AAA matrices) may be used as proteins descriptors. However, there is some loss of information associated with AAA, which records frequency of occurrence individual pairs of amino acids, but no information on their distribution.

I was interested to find out if one can introduce additional information on proteins in AAA matrix so that at least some lost information is recovered. It occurred to me that if in 2 × 2 AAA matrix instead of recording occurrence of each adjacent pair of AA one would write their sequential position, one would have information on distribution and abundance. By this, in fact we created novel type of matrices, to the best knowledge of mine, unknown in mathematics: matrices whose entries are not numbers but sets of numbers. However, most importantly in this way one would solve exactly the protein alignment for a pair of proteins. All that one has to do is to superimpose AAA matrices of two proteins, examine each (of 400) matrix elements and extract amino acids that have sequential neighbors in vicinity, collects all pairs which have the same sequential difference, and order them lexicographically. The resulting table represents the exact solution to the protein alignment problem, because it lists groups all pairs of AA which are at the same separation in the two proteins together. The extension of this approach to find the exact solution to the DNA alignment problem is straightforward.

SW06.W29–24
Reactome knowledgebase: annotating cancer variants and anti-cancer therapeutics
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Reactome (www.reactome.org) is an open-source, free access, manually curated and peer-reviewed biological pathway knowledgebase. Recent extensions of our data model accommodate the annotation of disease events, such as those associated with cancer. To support the graphical representation of cancer-related pathways, we have altered our Pathway Browser to display disease variants and events in a way that allows comparison with the wild type pathway, and displays connections between perturbations in cancer and other biological pathways. The curation of pathways associated with cancer, coupled with our efforts to create other disease-specific pathways, will interoperate with our existing pathway and network analysis tools. Previously, we have developed the Reactome FI CyberCope plugin, which can construct a FI sub-network based on provided gene list, perform network clustering analysis using a very fast, in-house implemented, modularity-driven spectral partition clustering algorithm, and annotate individual network modules using pathway/Gene Ontology enrichment analysis. A recent extension of Reactome FI network, with updated protein-protein interactions and other pairwise protein or gene relationships, genome-wide screening data sets, including interactions between transcription factors and their targets from the ENCODE project, and mouse protein-protein interactions has increased the FI network to covering just over half of human proteins in SwissProt and almost 274,000 interactions. We have also implemented the HotNet algorithm, for finding significantly altered sub-networks by using a graph heat-kernel model. The HotNet algorithm, combined with the survival analysis function and other previously implemented features of the Reactome FI plugin should provide a powerful framework for molecular signature discovery.

SW06.W29–25
Characteristics for sugar modifications extracted from protein tertiary structures
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In recent years, carbohydrate chains have been considered as ‘the third chains’ in life science. The glycobiology study had been expanded worldwide, through development of comprehensive analysis and synthetic technique of chemical structure, gene discovery of the glycosylation related proteins and functional analysis of carbohydrate chains. Bio-synthesized proteins are transported to specific organelles, receiving post-translational modifications (PTMs), and modified proteins express various functions. Glycosylations, one of the PTMs, are known that glycosyltransferases recognize specific motif sequences (N-linked glycans: Asn-X-Thr/Ser, O-linked glycan: Thr/Ser). Most glycoproteins and glycolipids were found in Endoplasmic Reticulum and the Golgi body. However, some glycosylations were known to occur in the nucleus and cytoplasm. Glycosylations are related to intra-cellular networks, due to controlling signal transduction systems and enzyme activities by cytoskeleton formation and ligand structural change, respectively. The difference of sugar types and modified positions, bringing variations of protein structures and functions, is caused by the selectivity of glycosyltransferases.

In this study, to find correlation between glycosylations and secondary structures, the three-dimensional coordinate data of
atoms in amino acids around the glycosylation sites were extracted from Protein Data Bank. The data was classified into sugar types. Propensities of relative side chain accessibilities and secondary structures were calculated by the three-dimensional coordinates from PDBFINDER2. The distances between glycosylation positions and each amino acid were calculated from the three-dimensional coordinate, and a tendency of atom and amino acid confirmed around a three-dimensional fringe area. As a result, N-glycosylation occurring in Endoplasmic Reticulum brings helical structure formation. On the other hand, it was suggested that there were few helical structures in O-glycosylation sites, because glycosyltransferases could not modify the sugars to helical structures. In addition, both ‘bend region’ and ‘random coil’ were found in N- and O-glycosylation sites frequently, and it was thought that glycosylations bring irregular structures according to increasing of solvent affinity.

**SW06.W29—26**

**Soy isoflavone, glycinin (4′-hydroxy-6-methoxyisoflavone-7-α-glucoside), promote human dermal fibroblast cell proliferation and migration by TGF-β signaling**

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Glycinin is one of soy isoflavones which are known to exhibit anti-oxidant, anti-allergic and anti-osteoporosis activities. In present study, glycinin was investigated for new activity promoting dermal fibroblast proliferation and migration. Treatment of glycinin to primary dermal fibroblast show dramatic increase of cell proliferation and migration. Twenty micromolar of glycinin treatment for 24 h induced the synthesis of collagen type I and III in both mRNA and protein levels. Fibronectin was increased by 20% after treatment. MMP-1, collagenase in the media was decreased after 24 h treatment of glycinin. The synthesis of TGF-β mRNA increased about two fold. The phosphorylation of Smad2 and Smad3 start to increase from 3 h treatment and continued phosphorylated to 24 h. The phosphorylation forms of AKT was increased after 3 h and continued to 24 h. Treatment of glycinin show anti-aging properties such as increase of total collagen in media, decrease of elastinase and decrease β-galactosidase in cells. The results indicate that glycinin stimulate TGF-β secretion and autocrine action of TGF-β provides proliferation and prevention against skin aging and wrinkling.

**SW06.W29—27**

**On the molecular basis of the onconase and barnase cytotoxicity**

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Ribonucleases (RNases) are the best-studied proteins in the world of biochemistry. Today, some of these proteins are giving new surprises. It is known that onconase and barnase are water-soluble proteins with antitumor action toward human tumor cell lines. Unfortunately, exact mechanism of the RNases cytotoxicity is still unclear. Here, the structural key, namely the hydrophobic (HΦ) segment, which allows to explain many biological effects of onconase and barnase, was reported.

Hydrophobic and electrostatic interactions play a general role when a protein locates near lipid bilayer, or at membrane interface. Modern views on membrane structure proposed that the surface of anionic membranes possesses a mosaic hydrophobic-hydrophilic nature [1]. This suggest that a protein, which have any hydrophobic structures, can be adsorbed by such hydrophobic regions.

The main aim of this investigation is to show that onconase and barnase have special hydrophobic properties, which participated in the apoptosis of tumor cells induction. Here, a clear evidence that onconase and barnase can be mimetics of proapoptotic Bax protein, which belong to the Bcl-2 family proteins, was obtained. It is may be useful for explanation of the mechanism of apoptosis induction.

Using a classical approach for determining the amphipilic helices was able to investigate the hydrophobic properties of onconase and barnase. By the helical wheel representation with calculating of the water-to-bilayer transfer free energy and hydrophobic moment values was demonstrated a special hydrophobic character of the helical structure elements of onconase and barnase. The results show a key role for the leucine residues in amphipilic helices of the toxic RNases. It was obtained that the leucine residue is exposed on the surface of above-mentioned proteins. What is it means for the cytotoxic RNases? This means that the HΦ segment is sterically accessible for hydrophobic interaction with a lipid bilayer. Further more, the values obtained allow to talk about thermodynamic favourability of the hydrophobic interaction.

Moreover, the results can rationally explain data by other scientists about the cytosolic internalization of onconase; it is known that the internalization of onconase is not dependent on a low pH environment [2]. These results show that onconase have a molecular determinant for such process, namely a helical HΦ segment formed by the helix I.

Natural function of barstar is to protect bacillar cells from the toxic action of barnase [3]. It is known that Lys-27 residue, which is locates in the helix II, participated at the interface of barnase-barstar complex [4]. The interface of barnase-barstar complex involves 45% of the residues in the interface are non-polar [5]. The knowledge about the HΦ segment, which formed by the helix II, suggests that barnase probably can to lyse a bacterial membrane in case of the lack of barstar.

Perhaps due to a HΦ segment some antitumor RNAses can interact with a lipid bilayer of the plasma membrane for adsorption, with the endosomal membrane for cytosolic internalization, and with the mitochondrial outer membrane for its permeabilization. Latter can be a cause of the apoptosis induction in tumor cells.

Using above-mentioned approach allows to define more precisely the thermodynamic parameters of the hydrophobic interaction of eosinophil cationic protein (ECP) with a lipid bilayer. It was shown that the first two N-terminal helices of ECP form a membrane-binding site [6]. The present work contains the exact thermodynamic parameters of these amphipilic helices, namely the water-to-bilayer transfer free energy and the hydrophobic moment values as a functions of α-helicity content, which was obtained from the in silico experiment. The results obtained can be useful for understanding of the bactericidal action of some secretory RNases.

Interestingly to hypothesize that toxic RNases with a HΦ segment can disrupt the tumor-derived microvesicles, or metastasomes, which contain different mRNAs, miRNAs, and oncogenic proteins [7]. The effective combination of membrane lytic and enzymatic activities suggests that toxic RNases can inhibit the tumor cell growth not only by apoptosis induction, but also by the alteration of intercellular signal pathways.

On the basis of these results, it may seem necessary to update modern views about the mechanism of RNase cytotoxicity. The results obtained suggest that the sequence of the HΦ segment of some toxic RNAses can be modified by replacement to hydrophobic amino acid residues. Such approach opens new possibilities in...
the rational design of novel antitumor drugs; some interesting methods already developed for well-known antitumor RNases, such as onconase, barnase, ECP, bovine seminal RNase, and binase. Further research will be focus on the dimer and lipid pore formation of the toxic RNases.

References


SW06.W29–28

Alpha-amylase – an enzyme present in various sequence-based glycoside hydrolase families

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Alpha-amylase (EC 3.2.1.1) hydrolises the alpha-1,4-glucosidic linkages in starch and related alpha-glucans. It is the best known amylolytic enzyme, generally possessing a broad substrate preference and product specificity. It is also one of the most frequently occurring glycoside hydrolases (GH) in the CAZy database, i.e. a sequence-based classification system of all Carbohydrate-Active enzymes. Alpha-amylase is found in families GH13, GH57 and GH119, and may be also in GH26. It is the main representative of the family GH13, which is well-known as the main alpha-amylase family, forming, together with families GH70 and GH77, the clan GH-H. Currently the specificity of alpha-amylase is assigned to several GH13 subfamilies (i.e. 1, 5, 6, 7, 15, 24, 27, 28, 36, 37 and, possibly, a few more not yet defined). The family GH13 alpha-amylases employ a retaining reaction mechanism, share 4–7 conserved sequence regions (CSRs) and adopt a TIM-barrel domain with the GH13 catalytic machinery. The alpha-amylases from the family GH57 use the same retaining mechanism, but they have their own 5 CSRs and catalytic machinery within an incomplete TIM-barrel fold. Interestingly, the family GH119 alpha-amylases likely share all above-mentioned features with the family GH57. Concerning the family GH126, the eventual presence of the specificity of alpha-amylase has to be confirmed by a more detailed biochemical characterization since the family GH126 representative exhibits a clear homology with GH families, the members of which are active to beta-glucans and employ the inverting reaction mechanism.
nucleotide atoms (bases, main chain). The more closely contacts are reproduced in the model the more accurate the model is considered to be. Contacts and contact areas are derived from the Voronoi diagram of spheres that correspond to heavy atoms of van der Waals radii. Dominating contacts in RNA are those between nucleobases. Therefore, we further distinguish stacking and non-stacking contacts between nucleobases. Interestingly, the contact area approach is able to closely reproduce base-stacking and base-pairing interactions despite a fairly simple definition. This is in stark contrast to previous approaches involving a considerable number of empiric parameters and thresholds.

We tested the new contact area-based evaluation method on a large number of RNA models and found that it is able both to effectively point out local errors in a model and to rank models by their overall quality. We believe that the new method should be useful both for the method developers and for the RNA community at large.

**SW06.W29–31**

Transmembrane region prediction – amino acid adjacency information based approach

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Transmembrane proteins play the central role in ligand transport across the biological membrane. Therefore, they are also potential drug targets. Despite the biological and pharmaceutical importance, transmembrane protein structures and functional mechanisms remain understudied due to experimental difficulties. Here, we present novel transmembrane region predictors for membrane proteins based on amino acid adjacency information using SVM classifiers. The prediction is based on the sequence information of the target protein. Since no homology information and evolutionary profile is required, prediction for transmembrane proteins with no significant homolog can be made with considerable accuracy. As the amino acid preference for secondary structures in transmembrane and globular domains can be similar, especially in case of β-transmembrane proteins, hydrophobicity or other physiochemical properties of the sequence is not considered. The prediction models have a two-layered architecture. The first is a SVM classifier based on mathematically encoded amino acid adjacency information of the sequence. It is trained on transmembrane domain information of proteins with known structures. The second layer makes the final prediction based on amino acid preference patterns at the transmembrane region boundaries, which are generated with statistical analysis of experimentally known transmembrane regions. We have developed two separate models for predicting transmembrane regions of α- and β-transmembrane proteins. Both are validated with sets of protein sequences that are not used in any stage of the algorithm development. The α-transmembrane region predictor shows a sensitivity of 89.83% and a positive predictive value of 91.7%. In case of the β-transmembrane region predictor, the sensitivity and the positive predictive value are 83.71% and 72.26%, respectively. The performances of the developed algorithms are also compared with other available predictors. Both our algorithms outperform the available predictors, including those developed in recent years based on sequence profiles. As the known transmembrane proteins do not yet sample the entire membrane protein space, our developed algorithms, independent of any evolutionary information, have an important advantage.

**SW06.W29–32**

Numerical analysis of gene networks models

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Gene networks are sets of genes functioning in a coordinated manner, their major components are the biopolymers DNA, RNA, and proteins. In this work, we consider a small network with several genes (they can be considered standalone) and observe the concentration of the corresponding proteins.

Studying the dynamic behavior of gene networks is a complicated problem. Therefore, we research the principles of their functioning on special hypothetical model. These models can be described by nonlinear system of differential equations.

We are very interested in the questions on existence and stability of attractors in such models. Particular attention is paid to some multidimensional symmetric systems with negative and positive feedbacks. These systems are studied with a specially developed algorithm, which reduces them to a single equation with delayed argument. Cycles in this equation searched by Andronov-Hopf theorem and stability analyzes using the first Lyapunov coefficient. Computer modelling and numerical experiments with our models have been implemented in our special computer program called PhasePortraitAnalyzer. The mathematical core is developed in R, and the interface part is created using C# + WPF. Its software can build the graphical representation of the gene networks phase portraits, search equilibrium points, simulate the trajectory and perform other calculations for analyzing the target system. We can prove the correctness of the numerical solutions using some theorems on topology of the models.

**SW06.W29–33**

A computational framework for de novo discovery of RNA editing events from RNASeq data

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RNA editing is a powerful instrument for the diversification of regulatory landscapes in eukaryotic genomes, promoting variability in the protein repertoire and silencing patterns, and presumably fighting the genome instability induced by mobile genetic elements. Recent advances in high-throughput sequencing technology have made it possible to study RNA editing on a genome-wide scale.

We developed a computational framework for de novo discovery of RNA editing events in RNASeq data sets, which aims to discriminate between the RNA editing signal and noise (e.g. sequencing errors, alignment errors, or SNPs) via estimation and comparison of their distributions in the RNASeq data set. The statistical model ensures high adaptability of the heuristic discovery method to noise behavior in sequencing experiment, which minimizes the number of potential false positive predictions. The approach is organism-independent and can be adapted to any sequencing protocol or type of sequenced RNA molecule.

An in-depth analysis of polyA-enriched RNA deep sequencing experiments in Drosophila tissues resulted in detection of a set of high-confidence A-to-I modification events in fly RNAs. Our approach enables de novo identification of the RNA editing events, which facilitates further functional studies of this critical step of post-transcriptional regulation.
Abstracts

SW06.W29–34
Evolutionary aspects of genome recombination in Densoviruses
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It is well known that recombination plays substantial role in generating genetic diversity that is a basis for adaptation, natural selection, and formation of new species. Recombination is as well one of the key mechanisms, beyond mutation events, of the development of new virus species, being the basis of their high variability. Thus studying recombination is essential for understanding the origin and evolution of different viruses as well as for virus systematic. In the presented work we first identified the recombination within densoviruses.

Densovirus (DNVs) (Densovirinae, Parvoviridae) is a highly diversified virus group infecting invertebrates. It includes four genera (Densovirus, Iteravirus, Brevidensovirus, Pefudensovirus) and a number of yet unclassified viruses. They are characterized by nonenveloped icosahedral capsid and a single-stranded linear genome. The DNV genome is subdivided into two halves encoding capsid (VP) and regulatory (NS) proteins flanked by noncoding terminal repeats.

First of all, we performed the phylogenetic analysis of DNVs based on the whole genome nucleotide sequences, and VP and NS aminoacid sequences. We showed that they correlated reasonably well, but came to conclusion that VP tree was more suitable for DNVs systematic as it represented more definitely the process of evolutionary development of densoviruses.

The investigation of recombination was assisted by RDP3 software. As DNVs are highly heterogeneous we were not able to detect recombination with reasonable level of significance when examining virus species from different genera. Therefore we restricted analysis to within groups of DNVs characterized by sufficient sequence similarity.

We showed that recombination occurred in Iteravirus genus leading to CeDNV origin from BmDNV1 and DpDNV. A number of recombination events were detected in Brevidensovirus, encompassing some DNVs infecting shrimps and mosquitoes, and in Densovirus genus. No significant events were detected inside the group comprising members of Pefudensovirus genus and a number of unclassified hemiteran and cockroach densoviruses.

The data obtained in the presented work are intended to further assist systematic of the densoviruses.

SW06.W29–35
Thrombin forms: bioinromatic analysis of structural data
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Thrombin – Na-dependent serine protease that plays a key role in blood coagulation cascade. Large amount of structural data was accumulated during long story of studies. Thrombin one of the most widespread proteins in PDB – there are 322 entries for human thrombin only. They consist of wild type and mutant proteins with varying length because of missing parts of flexible loops. However many of them are known to be almost identical in terms of Cα RMSD.

Considering that, the aim of the work is to find small number of reference structures that are able to cover all the conformational states of thrombin. Using similarity metric based on all-atom RMSD and Affinity propagation clustering algorithm, with a probability for a structure to become reference depending on resolution, we subdivided all the 364 individual thrombin molecules from PDB into 14 groups and extracted reference structures.

Nine groups are large, very uniform and represent thrombin in the active state. The 5 others are small (not >5 structures), distinctly differ primarily in the area of sodium binding site and active site cleft environment and represent conformational ensemble of thrombin in the inactive state.

Transitions between states corresponding to extracted references were observed by molecular dynamics simulations.

Obtained results can be used in the development of next-generation anticoagulants and in crystallographic studies.

SW06.W29–36
Serine/threonine protein kinases eukaryotic type: identification, classification, possible functions
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Eukaryotic-type serine/threonine protein kinases (ESTPKs) are the major participants in intracellular signal transduction and they are key players in many life processes in bacteria. We first carried a structural classification of 471 ESTPK of various gram-positive bacteria from 15 genera that live in the soil and in the gut microbiota, including the genus Bifidobacterium. ATP binding sites of kinases have been investigated and they were compared with homologous regions of the bacterial kinases with known crystal structure. Nine amino acid residues of adenosine binding pocket are important for the interaction were identified. Different combinations of these residues define the physico-chemical characteristics of the ATP binding pocket. Twenty groups of kinases that have a different set of these amino acid residues were identified. Bifidobacteria are an essential component of human gastrointestinal microbiota. The interest in bifidobacteria in connection with their probiotic properties and their positively influence on human health increased in recent years. We have identified six different ESTPK from Bifidobacterium distributed according to our classification into six different groups; five of these groups contain a representatives of only the genus Bifidobacterium. The bifidobacterial genomes of 48 strains of 11 species were studied; 261 ESTPK were found. All genomes of bifidobacteria contained five or six genes coding for ESTPK. Genes pkb1, pkb2, pkb3, pkb5 and pkb6 are present in all bifidobacterial genomes. The pkb4 gene was found only in B.longum and B.bifidum, as well as in B.breve UCC2003. The conserved genes pkb3, pkb5 and pkb6 are located in conservative areas relative to oriC. The pkb1 is located close to the area terC. The specific genes pkb2 in different bifidobacterial species are differently located on the chromosomal map relative to oriC and conservative ESTPK. However, their genetic environment is similar. The unique gene pkb4 within a species B.longum is conservative relative to other ESTPK and oriC. In species B.breve pkb4 is found only in one strain of B.breve UCC2003, and in its immediate genetic environment are two transposase gene, suggesting that its appearance is the result of horizontal transfer of gene.

Using similarity metric based on all-atom RMSD and Affinity propagation clustering algorithm, with a probability for a structure to become reference depending on resolution, we subdivided all the 364 individual thrombin molecules from PDB into 14 groups and extracted reference structures.
Docking of bacterial luciferase and NADPH:FMN-oxidoreductase using continuum electrostatic method

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Bacterial luciferase is a heterodimer (70 kDa) that catalyzes light-emitting reaction requiring long-chain aliphatic aldehyde, reduced flavin (FMNH2) and oxygen as the substrates. Along with luciferase luminescence system of bacteria includes a set of coupled enzymes that supply the substrates. Large fatty acid reductase complex synthesizes the fatty aldehyde for bioluminescence. The required FMNH2 is believed to be supplied by NAD(P)H-FMN oxidoreductases. Reduced flavin can be rapidly autoxidized, rendering free diffusion inefficient for in vitro intermolecular transfer. There are experimental evidences as to the complex formation for luciferase –oxidoreductase pair and independent work of these enzymes. Recently the crystal structures of bacterial luciferase and NADPH:FMN-oxidoreductase from Vibrio harveyi became available. It allowed analyzing a possible complex formation between these two proteins using computational methods.

The aim of this study was to find the evidence of bacterial luciferase – NADPH:FMN-oxidoreductase complex formation on the basis of molecular specificity and electrostatic potentials of this enzymes.

The approach for the docking study of this complex is based on continuum electrostatic model and the Poisson-Boltzmann equation. The result of the Poisson-Boltzmann equation is the electrostatic potential; its visualization can give first insights into the interaction between proteins. In the first step of the calculation, electrostatic potentials of both proteins were obtained. Then Monte Carlo sampling was used to generate docked complexes. Low energy configurations were accepted according to Metropolis criterion and 10 structures were analyzed in order to find the evidences for the complex formation.

The analysis of docked complexes revealed that bacterial luciferase and NADPH:FMN-oxidoreductase do not form a complex or the complex is very transient. The mobile loops closing the active sites in both proteins could play a key role for direct flavin transfer in the transient complex. The ARG291 and GLN197 from luciferase and oxidoreductase respectively are important aminoacid residues for such protein-protein interaction.

The work was supported by the grant No 11. G34.31.058 of the Government of Russian Federation.

In silico analysis of the conserved regions with stable secondary structure within the NS gene of human influenza A viruses

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Influenza A viruses (IAVs) cause an acute respiratory disease in humans, often resulting in severe epidemics and pandemics. Molecular mechanisms of pathogenicity of IAVs are poorly investigated. According to our previous and other research groups published data the dependence between the secondary structure of the viral genomic RNA and IAVs pathogenicity can be observed. Particularly we earlier identified two conserved 60–80 nucleotides long hairpin-like regions within the NS gene of human A/H5N1 viruses. The present study was focused on the search for the conserved stable hairpins in the secondary structure of NS gene of human IAV strains of six subtypes: H1N1, H2N2, H3N2, H5N1, H7N7, H9N2. On the base of global alignment of all genetically different sequences, made using MAFFT software, within the regions of the gene NS, specified as REF1 (80–150 bp) and REF2 (485–530 bp), the groups within each IAV subtype, comprised of only strains that differed in these regions, were formed. A/Xinjiang/1/2006 (H5N1) strain was chosen as a reference strain, for which stable secondary structures of both regions were predicted. Zucker algorithm was applied for the calculation of secondary structures and parameters using ViennaRNAfold software. According to the groups of the aligned sequences within each IAV subtype we have built the averaged secondary structures with possible mutational changes using RNAalifold software. It was shown that the characteristic secondary structures were predicted for both REF regions for the A/H5N1 viruses only, as well as for the second region A/H7N7 and A/H9N2 viruses. However, a more detailed analysis of each strain individually showed that the stable hairpin-like structures in REF1/REF2 regions were predicted for all IAV subtypes with a frequency of 25/67% for A/H1N1; 70/38% for A/H2N2; 30/23% for A/H3N2; 88/75% for A/H5N1; 50/100% for A/H7N7 and 50/50% for A/H9N2 viruses. It should be taken into account that all IAV strains H5N1, H7N7, H9N2 subtypes, isolated from humans, are mainly recognized as high-pathogenic, while a certain percentage of IAV strains of H1N1, H2N2 and H3N2 subtypes are pandemic. Thus the data obtained in this work allow to

Correlation between signal peptide sequences and physicochemical properties of mature formed proteins

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Biosynthesized proteins transported to the Endoplasmic Reticulum (ER) receive post-translational modifications through ER-Golgi networks and localize to their appropriate positions. Proteins become mature forms by receiving necessary post-translational modifications and fulfill their functions. Many proteins have signal regions which are encoded by 15–60 amino acids in N-terminus, including ER-targeting signal, ER-retention signal, nuclear localization signal, mitochondrion targeting signal. Particularly, many ER signals have been discovered because most proteins receive post-translational modifications in the ER. ER-targeting signals have high hydrophobicity due to being embedded in lipid bilayer. Signal-anchor and Signal-peptide mean ER-targeting signals which become transmembrane region or short peptide in mature formed proteins, respectively.

Various sequences of signal-peptides, consisting of 20–30 amino acids, have been identified, however consensus sequences of signal-peptides were not clarified. Different characteristics can be founded in the amino acid propensities of various signal-peptides, for example, high or relatively low hydrophobic residues.

In this study, physicochemical properties of human mature proteins which have signal-peptide in their full-length sequences were focused on, and correlation with signal-peptide sequences was extracted. First of all, the sequences of signal-peptide and mature proteins, information of protein function and subcellular localization were extracted from UniProt Knowledgebase/SwissProt release 2012_06. The characteristics including number of residues, molecular weight, size, hydrophobicity of mature proteins and signal-peptide sequences were analyzed. A correlation between signal-peptide sequences and properties of mature proteins will be reported in detail in the poster session.

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assume that the presence of specific secondary structures in the NS gene of human IAVs can be associated with the degree of viral pathogenicity.

### SW06.W29–40
**Amino acid preferences in extremophiles**

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Proteins of microorganisms living under extreme conditions such as high/low temperature, acidic/basic pH, high salinity or pressure receive increasing attention owing to their interesting properties. Such environmental extremities impose threatening stress on microorganisms. Since movement to a friendly environment is not easy, for survival under these stringent conditions microorganisms require adaptive changes. The ability of these organisms to survive under hostile conditions depends to a greater extent on the stability and integrity of their proteins. Here, we have investigated the correlation between protein amino acid composition and extremophilic properties. To this end, correspondence analysis has been used as a statistical tool to identify amino acid preferences of microorganism living in extreme environments. In order to classify microorganisms (bacteria and archaea) based on the distribution of amino acids, we have focused on the enzymes of the central catabolism (glycolysis, pentose phosphate pathway and TCA cycle) with the motive that these enzymes are highly conserved. Despite their differences, there was no distinctive separation between archaeal and bacterial proteins, but the differences between amino acids distributions reflected extremophilic properties clustering microorganism with similar properties together. This project has been supported by Marmara University Research Fund projects.

### SW06.W29–41
**Fatty acid regulation of gene expression: bioinformatics view to structure and dynamics of DNA-fatty acid complexation**


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The study of interaction between DNA and low molecular mass ligands using bioinformatics approaches is of great theoretical and practical interest for biochemistry and pharmacology. DNA is a target for peptide antibiotics and fatty acids, which bind specifically in the minor groove and take a part in regulation of gene expression. In this paper, the complexes of a DNA oligonucleotide consisting of 25 A–T base pairs (dA)₂₅·(dT)₂₅ and oleic or linoleic acid in neutral and ionized forms were studied by the molecular dynamics method with the use of an ‘explicit solvent model.’ When generating the DNA structure, we used the AMBER99 force field parameters. Ligands were characterized using General Amber Force Field (GAFF) parameters with AM1BCC charges. The molecular dynamics trajectory was calculated using the NAMD software package. After optimization of the complex of linoleic acid (EIC LA) and DNA, the number of interatomic interactions (van der Waals contacts) between the two structures was 106 (for oleic acid – 46). We selected three atoms in the EIC and three atoms in DNA that were the closest to the selected atoms of EIC. The atoms of the first pair (EIC41: H31–dT37: O1P) are located at a distance of 1.68 Å = hydrogen bond. The distance between the atoms in the next pair (EIC41: C10–dT34: H1') is 2.88 Å. This pair consists of the carbon atoms of EIC that form the double bond in the EIC itself and the hydrogen atom in the deoxyribose of pyrimidine. The distance between the penultimate carbon atom in the EIC and the hydrogen atom in the deoxyribose of pyrimidine (dT34: H5') is 2.96 Å. It is shown that these complexes have a high conformational mobility. The free energy of complex formation between DNA and linoleic acid (8 and 13 kcal/mol for the anion and acid, respectively), were determined. Two main conformations of the complex of linoleic/oleic acid with DNA were identified. In the course of conformational dynamics, the formation and destruction of interactions between the polar and nonpolar residues of linoleic/oleic acid, on the one hand, and DNA, on the other, were observed. Thus, an important role in a stabilization of DNA-fatty acid complexes play hydrogen binding, van der Waals and hydrophobic interactions.

### SW06.W29–42
**Molecular dynamics simulation approach for DNA duplex thermal stability prediction**

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Due to significant progress in development of computer software and hardware the *in silico* research became a useful tool to study biopolymers properties. Development of derivatives and analogues of nucleic acids remain laborious, costly and time-consuming. The using of computer simulations may allow precalculate physico-chemical properties of new derivatives before chemical synthesis. The aim of this work is to study a molecular dynamics approach for nucleic acid thermal stability calculation.

Using Amber 11 software (UCSF, USA) we simulated single and double stranded oligodeoxyribonucleotides. The enthalpies of DNA duplex formation were calculated as a difference of the total internal energy of double- and single-stranded states which were averaged from 10 ns MD trajectory. Computations were performed on NVIDIA GTX580/Intel i7-2600 hardware and resources of Siberian supercomputer center (ICMMG SB RAS). The use of GPU has speeded up the modeling in implicit solvent up to 60 times and up to 30 times in explicit solvent in comparison with the one node of CPU.

To determine optimal parameter set of modeling we have used Dickerson-Drew dodecamer (DDD) 5’-CCGGAATTCGCCG-3’ with well characterized secondary structure and thermal stability. We have varied force field, temperature, heating protocol, and ion concentration in implicit and explicit solvent, solvent shell radius and compared averaged double stranded DNA structures with those experimentally obtained. We have determined the optimal parameters of modeling in implicit and explicit solvent. It was shown that the experimental and obtained via the MD simulation conformations of duplexes structures are close to each other. Also the difference of experimental and calculated via the MD simulations enthalpies differ <15% whereas the experimental accuracy is about 10%.

To verify the MD predictive ability we have collected database of experimentally determined thermodynamic parameters (enthalpy and entropy) of hybridization of more than 300 oligodeoxyribonucleotides. The length of oligonucleotides varies from 4 up to 16 base pair (aver. 9 bp), GC-content 0–100% (aver. 57%). The total energy of oligonucleotide or duplex was averaged over 10 000 snapshots of 10 ns trajectories simulated with optimal parameter set. We have observed high correlation between the values of hybridization enthalpies obtained experi-
mentally and calculated using MD in implicit and explicit solvent. The best prediction of thermodynamic parameters was obtained in explicit solvent after analysis of 10 ns MD trajectories using Molecular Mechanics Poisson Boltzmann Surface Area (MMPBSA) calculations at 300K.

The RMSD and average error values of calculated and experimental enthalpies were <12 and 15%, respectively. The results obtained show that MD modeling allows one to calculate enthalpy of matched DNA duplexes with surprisingly good accuracy.

It is known that experimental enthalpies and entropies of DNA duplex formation are in a very good linear correlation ($R^2 > 0.99$). Based on this this dependence we have calculated entropy and free Gibbs energy of complexation. The average error of Gibbs energy prediction was 13%. Using these values we calculate DNA duplex melting temperatures. The average error of melting temperature calculation using molecular dynamics simulation was 4.6 °C. The results obtained is unexpectedly very good approaching to the widely used empirical nearest neighbor model allows to predict internal energies of DNA duplex formation and melting temperatures with accuracies 10% and 1.5 °C, respectively [1].

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Reference

SW06.W29–43
Possibility of the accurate prediction of secondary structures of loop regions in transmembrane proteins
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Transmembrane proteins act as core molecules in living cells including enzymes, receptors and channels like gateways which deny or permit the transportation of the specific substances across the biomembrane. Transmembrane proteins have membrane-spanning region(s) with high hydrophobicity, called ‘transmembrane region (TMR)’. On the other hand, the regions which are outside of biomembranes, usually the regions which are not TMRs and connecting each TMR, are called ‘loop region (LR)’. LRs are known as the interaction sites with other molecules and the post-translational modification (PTM) sites including glycosylation, lipidation and phosphorylation. Therefore, secondary structural information of LRs will help to predict PTM sites and ligand-binding sites and to analyze protein functions.

The number of 3D structural data of transmembrane proteins is much smaller than those of soluble proteins. Solubilization and crystallization are necessary in the structural analysis of transmembrane proteins, however it is difficult to keep their conformation as same as native state during the experimental process. Based on the above understanding, prediction of local secondary structures is essential in studies of 3D structure of transmembrane proteins, however it is difficult to keep their conformation as same as native state during the experimental process.

First, the sequence data of LD have the information about TM and secondary structure in the PDBFINDER2 database. The results of melting temperature calculation using molecular dynamics in normal and pathological conditions. The remaining part of Transposable Elements (TEs) make up almost half of the human genome sequence, and have played an important role in our evolution as well as in the development of various pathologies. Recent progress in Next-generation Sequencing (NGS) technologies is resulting in vast amounts of individual genomic data from patients suffering from various diseases, which gives a unique opportunity to analyse TE transposition dynamics in normal and pathological conditions.

Many methods for de novo discovery of insertions and deletions of TEs in sequencing data exist. However, most of them
Conclusions: At the transcriptional level in MPM, members of the two-pore-domain potassium channels are differentially expressed compared to healthy tissue. These results set the basis for the study of the pathophysiological role of KCNK-1, -3, -7 and -10 in MPM.

**SW06.W29-47**

Computational transcriptomic analysis of claudins in malignant pleural mesothelioma reveals significant correlations in their gene expression patterns

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Background: Claudins are involved in the formation of tight junctions that govern the intracellular connection of mesothelial cells in the pleura. They regulate the intracellular transport of water and solutes, pivotal function in the formation and preservation of the pleural membrane. Their contribution to malignant pleural mesothelioma (MPM) pathobiology is unknown.

Aim: The main goal of our study was to assess the gene expression levels of Claudins 1–18 in MPM as compared to controls.

Methods: We used gene expression data from the Oncomine Cancer Microarray database (http://www.oncomine.org/) comparing malignant pleural mesothelioma versus healthy tissue in order to investigate the differential expression profile of claudins 1–18. The gene expression data were log transformed, median centered per array, and the standard deviation was normalized to one per array. Gene expression was considered to be over- or under- expressed when its fold change in the MPM group was significantly different compared to controls (p < 0.05).

Results: In MPM, the gene expression of Claudin 15 was found to be significantly over-expressed in MPM patients (p < 0.0001). On the other hand, Claudins 4, 5, 7, 8, 9, 10 and 18 were significantly under-expressed compared to controls (p = 0.006, p < 0.0001, p < 0.0001, p = 0.032, p < 0.0001, and p = 0.005 respectively). On the contrary, there was no significant difference in the gene expression of Claudins -1, -3, -6, -7, -11, -14, -16, and -17. We also found significant positive correlations between the gene expressions of Claudin -4 and -16 (Pearson’s r = 0.48), Claudin -9 and -7 (r = 0.35), Claudin -9 and -11 (r = 0.32), Claudin -9 and -17 (r = 0.35), Claudin -9 and -16 (r = 0.35), Claudin -10 and -17 (r = 0.32), Claudin -18 and -14 (r = 0.50), Claudin -18 and -16 (r = 0.38), Claudin -15 and -18 (r = 0.80). Finally, there were significant negative correlations between the gene expressions of Claudin -5 and -8 (r = -0.33), and Claudin -5 and -6 (r = -0.45).

Conclusions: Our results indicate that members of the claudin family are differentially expressed in patients with MPM and that their gene expression patterns correlated in a high extend. The prognostic value of our findings requires further investigation.
SW06.W29–48
CLIC-3 and -4 genes are over-expressed in malignant pleural mesothelioma patients
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Background: Chloride Intracellular Channels (CLICs) are responsible for the regulation of multiple cellular functions. CLICs have been found over-expressed in several malignancies, and therefore they are currently considered as their potential as drug targets.

Aim: The main goal of our study was to assess the gene expression levels of CLIC’s 1–5 in malignant pleural mesothelioma (MPM) as compared to controls.

Methods: We used gene expression data from the Oncomine Cancer Microarray database (http://www.oncomine.org/) comparing malignant pleural mesothelioma versus healthy tissue in order to investigate the differential expression profile of CLIC 1–5. The gene expression data were log transformed, median centered per array, and the standard deviation was normalized to one per array. Gene expression was considered to be over- or under-expressed when its fold change in the MPM group was significantly different compared to controls (p < 0.05).

Results: In MPM, the gene expression of CLIC3 and CLIC4 are significantly increased compared to controls (p = 0.0014 and p = 0.0005 respectively). On the contrary, there was no significant difference in the gene expression of CLIC1, CLIC2 and CLIC5. We also found a significant positive correlation between the gene expression of CLIC3 and CLIC4 (p = 0.0016 and Spearman’s r = 0.48). Deming regression analysis revealed significant positive association (p = 0.0008) between the gene expression of CLIC3 and CLIC4 given by the following equation: CLIC4 = 4.42 CLIC3–10.1.

Conclusions: Our results indicate that CLIC3 and CLIC4 are over-expressed in human MPM. The prognostic value of CLIC3 and CLIC4 requires further investigation.

SW06.W29–49
Under-expression of ADRB1 and ADRB2 genes in malignant pleural mesothelioma
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Background: Beta-adrenergic receptors have been shown to be up-regulated in several malignancies. Their role has been mainly reported in cell proliferation and invasion and the administration of beta-adrenergic antagonists has been proposed as a therapeutic approach.

Aims: The goal of our study was to investigate of the gene expression levels of β1, β2 and β3 adrenergic receptors (ADRB1, ADRB2 and ADRB3) in malignant pleural mesothelioma (MPM) as compared to healthy controls.

Methods: We used gene expression data from the Oncomine Cancer Microarray database (http://www.oncomine.org/) comparing MPM versus controls in order to investigate the differential expression profile of beta adrenergic receptors. The gene expression data were log transformed, median centered per array, and the standard deviation was normalized to one per array. Gene expression was considered to be over- or under-expressed when its fold change in the MPM group was significantly different compared to controls (p < 0.05).

Results: In MPM, the gene expression of ADRB1 and ADRB2 are significantly decreased compared to healthy tissue (p = 0.015 and p < 0.0001 respectively). On the contrary, there was no significant difference in the gene expression of ADRB3 (p = 0.89). Deming regression analysis revealed a significant positive association (p = 0.048) between the gene expression of ADRB1 and ADRB2 given by the following equation: ADRB1 = 5.65 ADRB2 – 1.71.

Conclusions: Our findings suggest that at the transcriptional level in MPM, ADRB1 and ADRB2 are down-regulated. Their gene expression patterns seem to be associated. The pathophysiological basis of our findings requires further investigation.

SW06.W30 Systems Biology (VI-W30)

SW06.W30–1
A universal DNA-independent redox cell clock
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To better understand the temporal organization of the cell and its key mechanisms, we sampled organisms with sequenced genomes from all major branches of the Tree of Life and performed metabolic reconstructions of their genomes. We complemented the genome annotations of each protein sequence with the numbers of sulfur-bearing Cys and Met residues, as well as CxxC motifs. The comparative study of the reconstructions demonstrated that in all species from the smallest proteobacterium Caronella radii to the largest eukaryotes, transcription/translation pathways and housekeeping functions are liable to a tight regulation by the redox status of the thioredoxin system. The role of the glutathione system seems to be limited to protecting the cell from oxidative stresses.

The delivery of reducing equivalents from any known version of intermediary metabolism to the thioredoxin system is controlled by a very strong positive feedback affecting SH-groups of all key enzymes of the metabolism. This results in generating square-wave oscillations without direct involvement of gene expression and protein synthesis. These self-sustained oscillations serve as a timer separating the reductive (anabolic) and the oxidative (catabolic) phases of cellular metabolism that cannot occur at the same time. This mechanism is consistent with the thiol cycle by discovered by Louis Rapkine in 1931. Mathematical analysis of it predicts a wide range of phenotypic features that can be easily demonstrated experimentally. In particular, the core of the redox clock can be modeled in vitro with a small number of purified enzymes generating square-wave oscillations in the redox status of thioredoxin.

SW06.W30–2
Shotgun metagenomic analysis reveals metabolic diversity of electrogenic microbial community
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Microbial fuel cells (MFCs), an innovative technology for bioelectricity generation, exploit microbial catabolic activities to gen-

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erate electricity from a variety of biodegradable substrates, including complex organic waste. Given wastewater as an input and pure water and electrical power as output, MFC comprise an absolutely advantageous eco-friendly bio-electroremediation system.

We introduce the study of metabolic potential of electrochemically active microbial biofilm obtained from anode surface of 2 month working microbial fuel cell. Out of all functional categories we focused on chemical compounds metabolism since it relates to process of wastewater treatment and respiration since it relates to the process of electron transfer. The categories of stress response and virulence were also considered since they constitute the important characteristics of microbial consortia involved into bioremediation processes. To obtain an accurate picture of metabolic potential of studied microbial population we performed functional metagenome analyses accumulating evidences from multiple sources including SEED categories and KEGG maps.

**SW06.W30–3**

**A system wide simulation model of translation**

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We present a computational system wide simulation model of translation. This model implements all known mechanisms that are thought to be important for translation. At present it is parameterised for yeast using the the best known quantitative estimates for key aspects of the system, including tRNA abundances, ribosome affinities, decoding speeds and kinetic constants. The model can be easily re-parameterised for other organisms, including eukaryotic cells or bacteria.

Using this model we first demonstrate that quantitative estimates of key aspects of yeast transcription as they are reported in the literature are largely consistent with one another. We also show that the model can be used to predict the effect of changes to key-characteristics of translation. In the second part of the talk we demonstrate how the model can be used to generate new biological insights that would be difficult or impossible to obtain without a computer model. Finally, we present some novel insights regarding the origin of the codon usage bias in yeast.

**SW06.W30–4**

**Fibrinolysis wave propagation in a reaction-diffusive system**

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**Background:** Fibrinolytic system is responsible for blood clot removal after the coagulation occurred. It is one of the vital systems of the human homeostasis which starts at the same time as the clot starts to grow, and like the coagulation system is spatially nonuniform. The aspects of this system functioning in the presence of propagating clot are not clear.

**Objective:** In this work we evaluated the underlying mechanisms of fibrinolysis and coagulation system interaction, using a newly developed model of spatial fibrin clot growth and lysis.

**Methods:** A thin layer of unstirred human blood plasma contacting with the surface with immobilized tissue factor was monitored to register the fibrin clot growth and its lysis. Plasma was supplemented with streptokinase, urokinase or tissue plasminogen activator at different concentrations close to pharmacological to test their ability to cause clot lysis. Time when the clot started to grow and when it started to dissolve, and rates of these processes were used to evaluate the influence of plasminogen activators on the system of plasma coagulation and fibrin lysis.

**Results:** Fibrinolysis started in area of activation of coagulation and propagated towards the direction of clot growth, the rate of clot lysis depended on clot growth rate (clot growth/lysis rates were 65 ± 6/66 ± 7 μm/min without phospholipids; 91/93 μm/min with phospholipids 4[μM]). The time of fibrinolysis onset increased with the decrease of tissue plasminogen activator (TPA) concentration (1.7 ± 0.3 min for 20 μg/ml TPA; 29 ± 14 min for 0.75 μg/ml TPA). It was found that the high concentrations of plasminogen activators [TPA (5>5 μg/ml)] caused activation of coagulation system, increased the clot growth rate (from 48 ± 3 μm/min without plasminogen activators; up to 65 ± 6 μm/min with TPA 20 μg/ml) and reduced the time when clot started to grow.

**Conclusions:** A novel model was capable to detect spatial clot lysis and can be used for further investigations of clotting and lytic systems interaction. Clot lysis rate depended only on the rate of clot growth, but not on the type or concentration of the plasminogen activator. High concentrations of plasminogen activators enhance coagulation, increasing clot growth rate.

**Conflict of interests:** The study was supported by the RFBR grants 11-04-00303, 12-04-00652, 12-04-00438, 12-04-33055. MAP, FIA and AMS are employees and/or founders of HemaCore LLC.

**SW06.W30–5**

**Building a predictive model of translation initiator ability of 5′-UTR prokaryotic mRNA**

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Translation efficiency of mRNA depends on its 5′-UTR sequence. For prokaryotes such features as Shine-Dalgarno sequences (SD), A/U-rich sequences (A/U), secondary structures and start codons and known to affect translation efficiency. We analyzed 5′-UTRs of E. coli genes to reveal the distribution of these features among known genes. We compared computed frequencies of such features with translational efficiency data for building of 5′-UTR primary model. We determined that most frequent SDs and A/U Us are not most effective. Curiously AU content of an upstream region of the first genes in operons are higher than that for the following ones. We measured translation efficiency for the set of model mRNAs with same CDSs but different 5′-UTRs. Additionally we tested a set of natural 5′UTRs. Experiments were done in a system with internal control based on dual cerulean and red (CER/RFP) fluorescent proteins.

**SW06.W30–6**

**pH is not related to cellular proteome**

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Intracellular pH is a tightly regulated global physiological parameter in cellular systems. Much is known about active and passive mechanisms of the control of pH. However relationships between these mechanisms and their relative contributions are not yet fully understood. Given that proteins with multiple protonable groups exist in the cell in the concentration exceeding those of free protons by a few orders of a magnitude, we assessed the total buffering capacity of proteomes in several model cells.
We will show, by theoretical calculations, that buffering capacity of proteomes varies with the cell type, but generally its contribution to maintaining pH is negligible. For a comparison, we did potentiometric titration of yeast cytoplasm which revealed that inorganic phosphates have much greater contribution to cytosol’s buffering capacity than the proteome. Both theoretical and experimental results are in agreement and, at the same time, in a serious contrast to the textbook knowledge from which one learns that cell’s buffering capacity comes mainly from the proteome. These findings complement recent evidence for cell-wide signalling role of pH and suggest that proteomes serve as signal receivers rather than playing an active role in redox homeostasis.

For the red blood cells, which constitute an extraordinary case, we show that hemoglobin, while being the major buffering agent, contributes roughly two thirds of the total and ca. 75% of the intrinsic, non-carbonate erythrocyte buffering capacity – again substantially less that it was assumed previously. Other significant contributions come from 2,3-bisphosphoglyceric acid, ATP and inorganic phosphates.

In conclusion, the basic assumptions on the role of global regulators such as redox equilibria, temperature and others should be revisited in the light of accumulating whole-cell data as often their interpretation based on the 20th century data is misleading.

SW06.W30–7
Oligocene origin, Holocene diversification and refugia of the European paleoendemic Haberlea rhodopensis (Gesneriaceae)
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Background: The Balkan Peninsula is a centre of Southeastern European biodiversity, but very little is known about paleoenvironmental species of Gesneriaceae family in this region.

Aim: To investigate the origin and genetic diversity of Haberlea rhodopensis in Bulgaria.

Methods: Plants from 10 different localities of Haberlea collected in Balkan and Rhodope Mountains were studied by chloroplast microsatellite markers, trnL-F intron-spacer and nuclear ribosomal DNA internal transcribed spacer sequences.

Results: All individuals representing investigated populations of Haberlea had identical trnL-F sequences. The divergence time of Haberlea and the remaining European genera was estimated at 27.5 to 29.7 MY ago. Two ITS types were discovered: one with no base polymorphism, and one possessing four base polymorphisms. These data are in agreement with the increased oxidative stress present in the gut of Crohn’s Disease (CD) patients. On the contrary, an increase of TIMP-1 occurs in the fibrotic processes of these patients. Oxidative stress activates MMPs and induces their expression through the activation of transcriptional factors [2]. Previous data showed in ISEMFS of CD patients an increased intracellular oxidative state related to a decrease of GSH/GSSG ratio [1]. For this reason, we investigated in ISEMFS isolated from colon of patients with active CD, the production of MMP-3, TIMP-1 and their ratio in relation to oxidative stress and antioxidant activity of N-acetylcysteine (NAC), a precursor in GSH synthesis. Preliminary results show that in these cells MMP-3 levels increased, whereas no change was measured in TIMP-1 levels, consequently MMP-3/TIMP-1 ratio was enhanced. TNFα stimulation increased further MMP-3 production as compared to unstimulated cells. These data are in agreement with the increased oxidative stress present in CD-ISEMFS. Pretreatment with NAC induced a significant reduction of MMP-3 level and it was also able to remove TNFα stimulation. A direct effect of NAC on MMP-3 production in CD-ISEMFS was demonstrated. This event did not seem related to changes in GSH levels. Therefore, these data suggest that NAC, through its antioxidant property,
Abstracts

Moreover, we found a concomitant upregulation of miR-101, which is in agreement with high miR-101 levels in GDM HUVECs.

Conclusion: These data identify a signalling pathway that links hyperglycaemia to miR-101 and its target gene EZH2, suggesting new targets in the fields of gestational diabetes and EC dysfunction induced by hyperglycaemia in utero. We characterise a novel mechanism regulating hyperglycaemia-impaired angiogenesis, with the possibility of restoring the angiogenic capacity of GDM cells in vitro by blocking miR-101 and subsequently upregulating its target EZH2.

SW06.W30–11
Ultrastructural changes in the rat fallopian tubes with ageing
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The mammalian fallopian tubes have important functions in reproduction such as picking up the oocyte following ovulation and moving it into the region where the fertilization occurs. It also involve in the transport and stability of sperm, fertilization and early embryo development. In this study, age related ultrastructural changes in fallopian tubes were aimed to be determined. Ultrastructural changes was examined in fallopian tube tissue samples of young (22 days), prepubertal (4 weeks), adult (10 weeks), premenopausal (8 months) and old (24 months) female rats using electron microscopy. In young group development of ciliated and secretory cells was completed and junctions were observed between cells. There were mitochondria and endoplasmic reticulum tubules in cytoplasm. Cilia and microvilli were detected in epithelial cells of prepubertal group. The content of organelles increased in adult group and this situation was thought to be related with function of fallopian tubes in early embryo nourishment. Beginning with premenopausal group degeneration of mitochondria and apoptotic cells were detected in fallopian tubes of advancing age groups in accordance with decrease in reproductive ability.

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SW06.W30–12
Characterization of the endocannabinoid system in telomerase-immortalized human endometrial stromal cell line, St-T1b
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Endocannabinoids (eCBs) are endogenous signalling mediators that bind to selective membrane receptors, the cannabinoid receptors. These molecules along with receptors, transporters and respective metabolic enzymes form the endocannabinoid system (ECS) [1]. Several eCBs have been discovered with emphasis on anandamide (AEA) and 2-arachidonoylglycerol (2-AG). They are involved in several basic biological processes like energy balance, immune response and reproduction, including gametogenesis, implantation and parturition [2]. A well-coordinated process occurs during the secretory phase of the menstrual cycle and involves differentiation of endometrial stromal cells (ESC) into

References


SW06.W30–10
Phenotypic characterisation and molecular changes induced by gestational diabetes mellitus (GDM) on human umbilical endothelial cells: focus on the KDM2B/miR-101/EZH2 pathway
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Background and aims: Gestational diabetes mellitus (GDM) is characterised by maternal hyperglycaemia that is first recognised during pregnancy. GDM results in chronic foetal hyperglycaemia and hyperinsulinaemia, with consequent changes in foetal endothelial gene expression and endothelial cell (EC) function. We aim to determine whether exposure to a diabetic intrauterine environment alters human umbilical vein EC (HUVEC) function, with further investigation into microRNA (miR) expression and epigenetic pathways.

Methods: HUVECs were extracted from expecting mothers with and without GDM. We performed functional assays and focused miR and target gene analyses on extracted cell lineages. Finally, we also studied ‘healthy’ HUVECs that were grown in high glucose conditions.

Results: We observed a reduced capacity of closure on scratch assay, diminished capillary-like tube formation on Matrigel and decreased proliferation in response to foetal bovine serum (FBS), after a period of FBS-starvation, in the GDM-extracted lineages compared with the controls. On microRNA screening we found a tendency towards increased levels of miRs belonging to the miR-15 family. Specifically, miR-101 was significantly upregulated in the GDM-extracted lineages; hence we focused on this miR’s molecular mechanisms in our target cell lineages. There was a strong positive correlation between miR-101 levels and apoptosis (Pearson r = 0.9, p value < 0.001). There was a decreased expression of Enhancer of Zester Homolog 2 (EZH2) in the GDM-extracted lineages. Confirming previous reports which have validated EZH2 as a target-gene of miR-101 in HUVECs, we found their expression levels were negatively correlated. EZH2 mRNA levels correlated positively with KDM2B and VEGF-A; conversely miR-101 levels correlated negatively with KDM2B. These data are in line with the described KDM2B:EZH2/miR-101/EZH2 axis.

Anti-miR-101 treatment in GDM cells resulted in upregulation of EZH2, decreased apoptosis and an improved angiogenic phenotype, restoring the endothelial tube formation to the level seen in control lineages. As expected, in vitro exposure of ‘healthy’ HUVECs to high glucose significantly impaired cellular function.

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Anti-miR-101 treatment in GDM cells resulted in upregulation of EZH2, decreased apoptosis and an improved angiogenic phenotype, restoring the endothelial tube formation to the level seen in control lineages. As expected, in vitro exposure of ‘healthy’ HUVECs to high glucose significantly impaired cellular function.

Moreover, we found a concomitant upregulation of miR-101, which is in agreement with high miR-101 levels in GDM HUVECs.
decidual cells, which are precondition for the establishment and progression of pregnancy. We have previously reported that ECS machinery operates on rat decidual cells and that AEA has the ability to induce apoptotosis through CB1 activation [3,4]. However, little is known regarding the presence and function of the ECS in human decidual establishment and function. In this study, the presence of the ECS components in endometrial stromal and differentiated decidual cells were characterized. This was achieved by immunoblotting, immunocytochemistry and qRT-PCR experiments in immortalized human ESC line cell line (St-T1b) [5]. We showed that St-T1b express classical cannabinoid receptors at mRNA and protein levels. Interestingly, we also demonstrated that St-T1b cells have the capacity to synthesize and degrade AEA and 2-AG. In fact, they express the respective metabolic enzymes NAPE-PLD, FAAH, DAGL and MAGL. Altogether, decidual may modulate local levels of eCBs by regulating the corresponding metabolic enzymes. Additionally, it indicates human decidual cells as potential targets for exocannabinoids resulting from cannabis consumption.

We thank Dr. Birgit Gellens for kindly provide St-T1b cell line. BM Fonseca thanks Fundação para a Ciência e Tecnologia (FCT) for the Post-doctoral grant (SFRH/BPD/72958/2010) and the PhD grant attributed to MA Costa (SFRH/BD/70721/2010) and M Almada (SFRH/BD/81561/2011).

References

SW06.W30–13
'Reverse Warburg' phenotype and PK-M2: regulation of pentose phosphate pathway and implications for chemoresistance
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Several evidences have established that cancer cells undergo profound changes in their metabolism, beside and in synergy with microenvironment [1]. In particular, tumor cells use Warburg metabolism to support an efficient anabolism from glycolytic intermediates and to increase cancer proliferation [2]. In fact, cancer cells express the M2 isoform of piruvate kinase (PK), which shift glucose metabolism towards aerobic glycolysis. The redox inhibition of PK-M2 during oxidative stress is furthermore important to divert glucose flux to the pentose phosphate pathway (PPP) in order to generate NADPH [3]. Furthermore, the activation of the PPP has been correlated to multidrug resistance (MDR) phenotype [4]. We demonstrated that the reciprocal interplay between CAFs (Cancer Associated Fibroblasts) and prostate cancer (PCa) cells establishes a sort of ‘Cori cycle’ where respiring cancer cells upload lactate produced by glycolytic fibroblasts, to drive anabolic pathways and ATP production and thus undergoing ‘reverse Warburg’ phenotype [5].

Our aim is to understand the interplay among tumor microenvironment, oxidative stress and metabolic reprogramming of cancer cells, as well as their possible involvement in drug resistance. In this regard, we found that PCa cells resistant to docetaxel show a decrease of intracellular ROS (Reactive Oxygen Species), NADPH level and proliferation compared to control cells. These features are not associated with induction of activity or expression of glucose-6-phosphate dehydrogenase, the rate limiting enzyme of PPP, but are associated with an increased expression of Nrf2 (Nuclear factor erythroid 2 related factor 2), a transcription factor involved in antioxidant response. Furthermore, docetaxel resistant PCa cells undergo an escape from Warburg metabolism and the resistance to drug is enhanced by co-culture with CAFs. We propose that chemoresistance to docetaxel induces, as already reported for CAFs, a ‘reverse Warburg’ phenotype, which is not associated with increased proliferation, but with a drug resistance advantage. We speculate that the recovery of oxidative metabolism subtracts glycolytic intermediates from anabolic processes and from PPP, to drive ATP production. Hence, NADPH and ATP may be used respectively to counteract the oxidative stress induced by drugs and to fuel MDR transporters. In this context, we will study if PK-M2 may be the key switch between Warburg and ‘reverse Warburg’ metabolism, playing a relevant role for chemoresistance.

References

SW06.W30–14
Binding of the coagulation factor IXa to the membrane of activated platelets
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Introduction: Membrane phase of proteolitic reactions on the activated platelets plays an important role in the process of blood coagulation by increasing the rate of these reactions by several orders of magnitude. The enzymatic complexes of protrombinase and intrinsic tenase are assembled on the membrane of activated platelets to achieve this. Activated platelets include two procoagulant phosphatidylserine-positive (PS+) subpopulations (they strongly differ in the intracellular calcium concentration) and one PS-negative (PS−) non-procoagulant subpopulation. The binding of coagulation factor IXa (FIXa), a main component of intrinsic tenase, to the membranes of these three subpopulations were the subject of this study.

Methods: FIXa was covalently labeled with fluorescein. Washed gel-filtered platelets were activated at 2 x 100/ml with 100 nM thrombin in the presence of 2.5 mM CaCl2 for 15 min. They were incubated with different concentrations of fluorescein-labeled FIXa and analyzed with a FACS Calibur cytometer. The surface distribution of FIXa on the activated platelets was also imaging using confocal microscopy.

Results: The addition of different concentrations of labeled FIXa to the three subpopulations demonstrated two types of binding with regard to the amount of bind factor per platelet. Two PS+ subpopulations showed insignificant difference (1.3-fold) in their FIXa binding. Binding to the PS-negative subpopulation was ~12-fold smaller in comparison with the PS+ platelets.
Generally, for both PS+ subpopulations the dependence of the bound factor quantity binding on the added FIXa concentration was linear without any saturation up to very high concentrations of FIXa (2000 nM, which is by several orders of magnitude higher than the physiological value). Confocal microscopy showed that FIXa localized on the surface of the PS+ platelets to a some ‘hat’-shaped formation. This localization could be important for additional acceleration of coagulation reactions. **Conclusions:** Two PS+ subpopulations of platelets is better than the PS- in binding of FIXa by one order of magnitude, their dependence of binding on the concentration of free FIXa is linear and without saturation. This suggests their major role in binding of FIXa during the clotting. Non-uniform, localized distribution of the FIXa on the surface of PS+ platelets, it’s ‘hat’-shaped formation, suggests that such a colocalization with other factors could work for acceleration of coagulation reactions.

**SW06.W30–15**

**New concepts about fibroblasts trophic function**

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In pluricellular organism, intercellular communication is instrumental for the survival and the function of cells and to ensure the integrity of tissues. The exchange of information could occur through the transmission of electrical or chemical signals or through the transfer of portions of cell membrane, either after direct cell-cell contact (mechanisms of trogocytosis, nibbling and nanotubes) or by the secretion of small vesicles composed of a lipid bilayer containing transmembrane protein and enclosing soluble molecules [1].

By cytfluorimetry, confocal microscopy and radiolabelled protein experiments we found that primary human fibroblasts transfer both proteins and lipids to cancer cells and to non-transformed cells. On the contrary, various lines of cancer cells are not able to perform this kind of effect, so this phenomenon is mainly unidirectional. Time-lapse confocal microscopy studies and radiolabelled protein experiments showed, respectively, that the passage of lipids and proteins could be mediated by cell-cell contact and/or through the transfer of small vesicles.

These data in addition with proliferation tests, where we have shown that cancer cells increase their growth rate of 30–40% when co-cultured with primary human fibroblasts, suggest a novel role of stromal cells in the context of tumor microenvironment, that could represent a general property related to the trophic function of connective tissue. In fact, in the simplest hypothesis, the proteins and lipids transfer could not only promote cells survival but also enhances cells proliferation by increasing the rate of mass accumulation to the lower limit necessary for cell division.

**Reference**


**SW06.W30–16**

**Results of the long-term observation of the population of Blumeria graminis f.sp. hordei in Latvia and Lithuania**

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Local populations of Blumeria graminis f.sp. hordei, causal agent of barley mildew, are very changeable due to migration, mutation, recombinations and direct selection, in result new dangerous pathotypes could spread rapidly. Therefore monitoring of racial composition of pathogen population is crucial to create effective plant protection systems, including resistant varieties. During last decade we analysed more than 2500 isolates of B. graminis f.sp. hordei collected in conidia and/or cleistothecia stages both in Latvia and Lithuania. For determination of virulence genes each single colony was tested on a set of differentials with different powdery mildew resistance genes, comprised 10 near-isogenic Pallas lines, barley line SI1 and varieties ‘Stelli’, ‘Goldie’ and ‘Meltan’. Significant differences between samples of the pathogen population were detected for frequencies of virulence genes Val, Va3 and Val3. During previous years a clear tendency to increase of mentioned virulences was observed in South-Eastern part of Latvia. At the moment, corresponding resistance genes are still effective in the Central and Eastern Asia, for example, in winter barley regions of China. Barley mildew resistance genes Mlo6, Mlo7, Mlo9, Mlo12, Mkl and Mlala can be recognised as unnecessary in Latvia and Lithuania conditions, because frequencies of corresponding virulence genes were high. Shannon- and Simpson index were calculated to describe variability in samples of the pathogen, as well genetic distance between populations was determined. Significant differences in diversity of virulence genotypes among regional sub-populations occurred; highest level of diversity was detected in the South-Eastern part of Latvia. Presented data could be used for elaborating the best strategy for resistance breeding under Latvian conditions.

**Conclusions:**

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**SW06.W30–17**

**Interactions between nanoparticles and calli cultures of red clover and flax**

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Until now the influence of nanoparticles (NPs) on organisms at the molecular level is questionable, especially their molecular regulation mechanisms. Calli culture is an important tool in plant biotechnology, which is used in various ways, such as organogenesis, somatic embryogenesis and generation of somaclonal variation. This study was aimed to analyse the effect of NPs on calli DNA methylation, somaclonal variation and cell ploidy. We examined the influence of variable concentrations of Ag, Au, Zn, Fe, Ni and C nanoparticles on calli tissue. In this work, embryos were grown on Murashige and Skoog medium with different concentrations of NPs suspension. Latvian origin flax accession ‘Blue di Riga’ and ‘Lirina’, red clover variety ‘Skriveru agrais’ was used for calli formation. Genes rich with CpG sites (26S ribosomal RNA gene, 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2) were analysed for determination of DNA methylation in calli cells using pyrosequencing method. Analysis of methylation level in the six CpG sites in calli revealed significant differences between control calli and calli grown on medium supplemented with NPs. Genetic diversity of calli cells is expressed mostly in different ploidy, i.e. calli cells have different number of chromosomes. Calli were analysed by flow cytometry techniques. The results revealed that there are differences in development of calli, ploidy changes in calli cells which are caused by different NPs on the cultivation medium. Cell ploidy variation in calli significantly depends on the dose of carbon NPs concentration in medium. nuDNA regions of pectin methylesterase (pme3) and Mlo-like
protein genes were analysed for somaclonal variation estimation. Sequences from both regions were analysed and differences between calli grown on different MS were detected. Financial support for this study was provided by the ERDF project ‘Plant breeding technology development on the biocompatible microchips’ (2010/0321/2D/1.1.1.0/10/APIA/VIAA/144).

SW06.W30–18
Investigation of the fructose uptake system in halophilic bacteria *Halomonas smyrnensis* AAD6T

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*Halomonas smyrnensis* AAD6T is a gram-negative, aerobic, exopolysaccharide-producing, and moderately halophilic bacterium (Poli et al., 2013) that produces levan, a fructose homopolymer with many potential uses in various industries (Poli et al., 2009). Levan is an example of exopolysaccharide which is homopolymer of fructose with many outstanding properties like high solubility in oil and water, strong adhesivity, good biocompatibility and film-forming ability (Kazak et al., 2010). The results of the recent study on the whole genome sequencing of *H. smyrnensis* strain AAD6T (Sogutcu et al., 2012) allowed the investigation of the *Halomonas smyrnensis* metabolic network, and these analyses indicated the importance of fructose uptake mechanisms for levan biosynthesis. Therefore, in the present study, the fructose uptake system of *H. smyrnensis* AAD6T was investigated. In *H. smyrnensis* AAD6, fructose uptake was carried out with a phosphotransferase system (PTS), which is a distinct method used by bacteria for sugar uptake where the source of energy is obtained from phosphoenolpyruvate (PEP). It is known as a multicomponent system that involves enzymes of the plasma membrane and those in the cytoplasm. The system (PTS<sup>fru</sup>) consists of enzyme IIB and enzyme IIC components which act as coworkers of the same protein, fructose specific phosphocarrier protein-HPr (Histidine protein, heat resistant) and a fructose-specific enzyme II A which generates fructose-1-phosphate as the cytoplasmic product of the PTS-catalyzed phosphorylation reaction. The fruA operon of *H. smyrnensis* AAD6 consists of complex of EIIIB<sup>fru</sup> and EIIC<sup>fru</sup> domains. EIIIB<sup>fru</sup> phosphorylates the carbohydrate bound to EIIC<sup>fru</sup>. FruA thus transports extracellular fructose through a phosphorylation-dependent process to yield fructose-1-P (F1P), which is then channeled towards to yield fructose-1,6-bisphosphate (FBP). The second component of the PTS<sup>fru</sup> is FruB, a fusion of EIIA, HPr, and EI modules into a single polypeptide, the last domain of which is responsible for conveying high-energy phosphates from phosphoenolpyruvate (PEP) into the system (Chavarría et al., 2012). The fru operon is regulated by a repressor, FruR. Characterization of the fructose uptake mechanism may give new possibilities in designing engineering strategies for enhanced levan biosynthesis by *Halomonas smyrnensis* AAD6T. This research has been supported by TUBITAK (MAG/110M613).

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SW06.W30–19
A model of ‘parasite–host’ relationship: could it be generated?

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There are many ways how to survive and spread successfully. It doesn’t matter which organism species are investigated, because an organism surviving and spreading is impossible without interaction with each other. The organisms’ interaction mechanisms are the main driving force of evolution. One of the complicated interaction mechanisms is the ‘parasite–host’ surviving strategy where a parasite plays an important role in the regulation of host population by affecting hosts physiology, anatomy, immunology, behavior and so on in different combinations. The different kinds of influencing strategies of ‘parasite–host’ interaction mechanisms are more complex for parasites with indirect life cycle. In order to survive and spread as far as possible parasites with indirect life cycle develop individual relationships with each of their hosts. Each stage of parasite life cycle has unique ‘parasite–host’ interaction mechanisms and can be investigated separately, but cannot be considered without understanding the whole parasite life cycle. There are many species of parasites with different life cycles. It is not always possible to investigate the life cycle of every species separately because of many factors (lack of time, resources, availability of material etc.). Thus, the understanding of relationships between host organisms and their relevant parasites on different stages of life cycle, and the general mechanisms of parasite evolution, allows to generate the models of ‘parasite–host’ relationships. The created models will predict possible interactions between a parasite and its host which have not been investigated before in the context of related species. Therefore by investigating one parasite life stage the data can be acquired almost about whole ecosystem, and not only about a limited research area.

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SW06.W30–20
Coordination of the human antiviral transcriptional program by stochastic interchromosomal interactions

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Gene transcription is a stochastic process because most of the proteins required to regulate this process exist at limiting concentrations. One of the best characterized examples of stochastic transcriptional activation is the virus infection-induced expres-
sion of the human IFN-b gene. Transcriptional activation of the IFN-b gene is a biphasic process requiring three distinct sets of transcription factors bound to the enhancer. During the early phase of virus infection, the limiting transcription factor NF-kB is captured by three defined genetic elements termed NRCs (NF-kB Reception Centers) in a small percentage of infected cells, and subsequently NF-kB is delivered to a single IFN-b allele only via interchromosomal interactions, thus triggering enhanceosome assembly and monallelic gene expression. The produced IFN-b protein amplifies the infection signal by stimulating expression of the IFN-b gene from both alleles and in a larger fraction of the cell population. We have identified 41 additional NF-kB regulated genes that are affected by NRCs. DNA FISH experiments revealed that the NRCs associate with each one of these genes, and this association correlates with their stochastic monallelic RNA expression. Remarkably, we found that each expressing cell organizes 2-4 NRC conglomerates in which many virus-induced genes are recruited to receive NF-kB and initiate monallelic gene expression. Single cell RT-PCR analysis, RNA/DNA FISH and in situ RNA hybridization experiments verified these data by showing that the NRC-regulated genes are expressed simultaneously in the same cell and in a stochastic manner. Taken together, these and other experiments strongly suggest that stochastic patterns of gene expression are coordinated by interchromosomal interactions occurring in a small proportion of cells.

SW06.W30–21 Development of insulin resistance under consumption of high-calorie diet in rat
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Obesity is a multifactorial disease characterized by excessive accumulation of adipose tissue as a result of sedentary lifestyle and consumption of high-calorie food. In its turn, obesity can lead to coronary heart disease, hypertension and type 2 diabetes mellitus, characterized by increased concentrations of blood glucose and glycated hemoglobin and insulin resistance. According to WHO, 80% of obese people have a prediabetic condition, but the mechanisms of its occurrence and progression are poorly understood. Therefore the aim of this work was to determine glucose, glycated hemoglobin and insulin blood levels, and to conduct the insulin resistance test in rats under consumption of high calorie diet.

The study was performed on white nonlinear rats with an initial weight of 90–100 g. During the first week, all rats received standard food ‘Purina rodent chow’ and water ad libitum. On the 8th day animals were randomly divided into two groups. Animals of the control group for 10 weeks received standard food, experimental group were on a hypercaloric diet. The concentration of whole blood glucose was determined by the device ‘HYUKOFOT-II’ (Ukraine) according to its instructions. Blood glycated hemoglobin concentration was measured spectrophotometrically using kits of reagents produced by Lachema® (Russia). Serum insulin levels were determined using ELISA. To confirm the condition of insulin resistance in experimental animals an insulin-tolerance test with own modifications was conducted.

Studies showed an increase of glucose blood level at the 10-th week of hypercaloric diet in 1.7 times compared with the control group. Concentration of glycated hemoglobin increased by three times compared with the group of animals that consumed a standard meal. Insulin levels in serum increased by 2.4 times compared with the control group of animals. Insulin-tolerance test showed slowing glucose uptake by insulin in animals that consumed a high-calorie diet.

Thus, the study shows the development of a prediabetic state in rats which were fed a high-calorie diet, as evidenced by increasing concentrations of glucose and glycated hemoglobin and development of insulin resistance, resulting in decreased sensitivity of peripheral tissues to insulin action and increasing by compensatory mechanisms, concentration of insulin in blood.

SW06.W30–22 DNA repair in Mycoplasma gallisepticum
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DNA repair is essential for the maintenance of genome stability in all living things. Genome size as well as the repertoire and abundance of DNA repair components may vary among prokaryotic species. The bacteria of the Mollicutes class feature a small genome size, absence of a cell wall, and a parasitic lifestyle. A small number of genes make Mollicutes a good model for a ‘minimal cell’ concept. In this work we studied the DNA repair system of Mycoplasma gallisepticum on genomic, transcriptional, and proteomic levels. We detected 18 out of 23 members of the DNA repair system on a protein level. We found that abundance of the respective mRNAs is less than one per cell. We studied transcriptional response of DNA repair genes of M. gallisepticum at stress conditions including heat, osmotic, peroxide stresses, tetracycline and ciprofloxacin treatment, stationery phase and heat stress in stationery phase. Based on comparative genomic study, we determined that the DNA repair system M. gallisepticum includes a sufficient set of proteins to provide a cell with functional nucleotide and base excision repair and mismatch repair. We identified SOS-response in M. gallisepticum on ciprofloxacin, which is a known SOS-inducer, tetracycline and heat stress in the absence of established regulators. Heat stress was found to be the strongest SOS-inducer. We found that upon transition to stationary phase of culture growth transcription of DNA repair genes decreases dramatically. Heat stress does not induce SOS-response in a stationary phase.

SW06.W30–23 Melatonin prevents rat kidney injury caused by exposure to aluminium
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Aluminium (Al) exists naturally in air, water, soil and also in our diets. This metal that has the potential toxic effects can be absorbed into the human body and accumulates in different tissues. Aluminium-induced toxicity might be due to the increase in oxidative stress. Melatonin is a neurohormone synthesized and secreted by the pineal gland which has strong antioxidant and has free radical scavenging properties. The aim of this study is to determine the role of melatonin against aluminium-induced renal toxicity in rats by microscopically and biochemically. Additionally our aim is to show that effects of Al and also melatonin on metallothionein that is antioxidant agent to oxidative stress by immunohistochemical analysis. Wistar albino rats used in this study were divided into five groups. Group I: control, group II: melatonin control, group III: animals injected subcutaneously
10 mg/kg melatonin, group IV: animals injected intraperitoneally 5 mg/kg aluminum sulfate (Al₂(SO₄)₃), group V: animals injected Al₂(SO₄)₃ and melatonin in same time and doses. All groups were injected three times per week for 1 month. Tissue samples from kidney were fixed in Bouin’s fixative for morphological studies and were fixed in formalin solution for metallothionein immunohistochemistry, and embedded in paraffin. Kidney tissues were homogenized in cold 0.9% NaCl by means of a glass homogenizer in order to obtain a 10% (w/v) homogenate. The homogenates were centrifuged. The clear supernatants were used for protein and enzyme analysis by spectrophotometrically. Administration of Al caused degenerative changes on renal tissues. When melatonin was applied the parameters of metallothionein immunoreactivity, which increase in aluminum group, were decreased. In Al group, serum urea, creatinine, uric acid, lipid peroxidation, xanthine oxidase activity and protein carbonyl levels increased. Treatment with melatonin reversed all these effects. These results demonstrated that administration of melatonin is a potentially beneficial agent to reduce renal damage in Al-induced renal toxicity.

**SW06.W30–24**

Melatonin is a potent modulator of antioxidative defense and cell proliferation against aluminum toxicity in rats

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Aluminum toxicity is associated with induction of oxidative stress in various tissues. Melatonin have been demonstrated in the gastrointestinal tract, where this molecule is present in amounts exceeding those found in the pineal gland by several-hundred-fold. The goal of the present study was to assess the toxic effects induced by aluminum in small intestine, as well as the role of exogenous melatonin on antioxidative defense and cell proliferation. Male Wistar albino rats used in this study. Aluminum toxicity was induced by injection intraperitoneally of 5 mg/kg aluminum sulfate (Al₂(SO₄)₃), three times a week for 1 month in rats. Melatonin injected subcutaneously at 10 mg/kg doses three times a week for 1 month. Samples from small intestine were fixed in formalin solution and embedded in paraffin for Ki-67 immunohistochemistry. Intestinal tissue was homogenized in cold 0.9% NaCl by means of a glass homogenizer in order to obtain a 10% (w/v) homogenate. The homogenates were centrifuged. The clear supernatants were used for protein and enzyme analysis by spectrophotometrically. Intestinal superoxide dismutase, glutathione peroxidase, Na⁺/K⁺ATPase activities and cell proliferation index were decreased and, lactate dehydrogenase, xanthine oxidase activities were increased in aluminum group. Treatment with melatonin reversed these effects. As a results, we can say that melatonin protects small intestine against aluminum toxicity through its antioxidant and proliferative effects.

**SW06.W30–25**

The studying of biogenesis process of outer membrane vesicles produced *Lysobacter* sp. XL1

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Gram-negative bacteria produce outer membrane vesicles (OMVs) containing biologically active proteins. There is a lot of information available about its structure and diverse biological functions. However, the question connected with the biogenesis of bacterial OMVs is still not clear. The gram-negative bacterium referred to as *Lysobacter* sp. XL1 secretes five bacteriolytic enzymes (L1–L5) into the culture medium. Bacteriolytic endopeptidase L5 is one of the most studied extracellular proteins of the bacterium. Enzyme L5 was shown to secrete into environment with OMVs. We have proposed that secreted endopeptidase L5 may affect the biogenesis of OMVs produced by *Lysobacter* sp. XL1. To confirm the assumption we divide OMVs obtained from liquid cultures of *Lysobacter* sp. XL1 using sucrose density gradient centrifugation. Then we analyze the obtained fractions by SDS-PAG electrophoresis and western blotting assay. We have found that OMVs containing endopeptidase L5 are localized within lighter fractions.

The data obtained for recombinant enzyme L5 in *Pseudomonas fluorescens* Q2-87/B also support the hypothesis. The culture of *P. fluorescens* Q2-87/B has been found to form more quantity of OMVs having a wider range of size in comparison with the culture of *P. fluorescens* Q2-87. In addition, we have studied the spectrum of lytic activity of OMVs produced by *P. fluorescens* Q2-87/B against conditionally pathogenic gram-positive and gram-negative bacteria and fungi. Then we have determined the lytic activity of these against *Staphylococcus aureus* 55 (MRSA) and *Bacillus anthracis* 71/12. The data obtained can help study artificial vesicular structures – liposomes – as a more effective antimicrobial preparation.

**SW06.W30–26**

Dynamics of the Elowitz et al. synthetic repressilator as a function of temperature

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One of the aims of Synthetic Biology is the development of genetic circuits capable of performing tasks such as selecting a state from two or more possibilities, count time, and act as a filter of a signal. In the case of genetic clocks, one important property is the degree of robustness of the length of the period of oscillation as a function of environmental conditions. One variable that is expected to affect this feature is temperature. Here, we study the effect of temperature on the dynamics of the repressilator, which comprises of three promoters connected together in a negative feedback loop. It has been shown that, at 30°C, the system produces periodic oscillations and the state of the network is carried to the daughter cells. We observed an apparent increase in the period when the temperature was increased. A mathematical model of the system was then used to explain the observations. From the comparison between model and measurements, namely, from the assessment of the rate of failure in oscillating of the real system, we conclude that the observations can be explained by an increase in the fraction of
failed oscillations which causes an apparent increase in period length. The decrease of robustness of this circuit with increasing temperature is in agreement with observations of the effects of this change on the degradation rate of the protein CI, one of the component proteins of the repressilator. The outcome of our study should aid in the future engineering of more robust synthetic genetic clocks.

**SW06.W30–27 Mathematical modeling of gene network dynamics in E. coli**

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Advance in genomes sequencing of the various organisms promotes the emergence of actual challenge that is high throughput functional annotation of genetic programmes encoded in these genomes. In this regard, the in silico approach is becoming more and more popular in the systems biology era. The method has almost unlimited research and predictive capacity. Especially acute the need is being under effort to create the full-scale model for a cell, for example, E. coli that is classic model object. The basic principles of structural and functional organization of the genome have been identified and the mechanisms of the basic fundamental cellular processes have been studied precisely on E. coli.

But to solve the most important task, first of all, it is necessary to create and continuously develop: (i) the modern computational approach for analysis of complex hierarchical organization of gene networks and for decomposition them into elementary subsystems; (ii) basic mathematical models of these elementary subsystems; (iii) different databases to accumulate and systemize the information about structural and functional organization of the systems, dynamics of their functioning and kinetic parameters for these processes.

We have presented a hierarchical modular approach for reconstruction of mathematical models describing gene networks functioning on the example of the pyrimidines biosynthesis pathway in E. coli. We have also demonstrated own databases on mathematical models and kinetic data for E. coli. The mathematical model of ribopyrimidines biosynthesis in E. coli was developed and numerically explored. The model takes into account nonlinear effects of the negative regulation of the enzymes activity by the end products. These enzymes catalyze the earlier stages of the process. The mechanisms and parameters of the enzymatic reactions were clarified. It was theoretically shown that the biological system has oscillatory regime of dynamical functioning.

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**SW06.W30–28 Ack1 overexpression induces dendritic arborization**

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Ack1 (Activated Cdc42-associated tyrosine kinase) is a non-receptor tyrosine kinase that is expressed in the brain. Here we show that Ack1 that in response to neurotrophins Ack1 interacts with Trk receptors and becomes tyrosine-phosphorylated. In addition, pharmacological blockade experiments indicate that Ack1 acts upstream of the Akt and MAPK pathways. We also show that Ack1 overexpression induces neuritic outgrowth and promotes branching in neurotrophin-treated neuronal cells, whereas the expression of Ack1 dominant-negatives or shRNAs counteract neurotrophin-stimulated differentiation.

**SW06.W30–29 Role of GSH and estrogen on serum-starvation induced apoptosis in osteocytes**

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Bone is able to repair microdamage through targeted remodeling process in which osteocytes are involved. Osteocytes are the major mechanosensory cells in bone and their apoptosis has been observed in vivo in microdamage and mechanical disuse. Therefore, microdamage-induced osteocyte apoptosis is due to reduced nutrient and oxygen transport and has been related to increased local bone turnover and resorption. Various data suggest that osteocytes through the apoptosis and the production of cytokines are regulators of bone remodeling. This study was performed in MLO-Y4 osteocyte like cells to evaluate the effect of glutathione (GSH) and estrogen on osteocyte apoptosis due to bone microdamage in relation also to hydrogen peroxide production. The mechanisms which regulate these events are not well known. In this study, serum starvation was chosen as apoptosis inducer; in fact, this method mimics osteocyte apoptosis induced in vivo by reduced nutrient transport in microdamage bone, as also reported by others [1].

Preliminary results show that in MLO-Y4 cells the starvation induces a time dependent apoptosis. Under the same experimental conditions it is also measured a significant increase in hydrogen peroxide levels. Apoptosis and oxidative stress are increased by cell pretreatment with butionine sulfoximine (BSO), a specific inhibitor of GSH synthesis. On the contrary, estrogen reduces apoptosis, but this effect was not due to its antioxidant property, as previously shown in these cells in which the apoptosis was induced by oxidative stress [2]. Possible mechanisms related to the activation of mitogen activated protein kinases or transcriptional factors and involved in the GSH and estrogen action were also investigated.

These data suggest that serum starvation-induced apoptosis in MLO-Y4 cells may be a redox regulated process with the involvement of GSH. Estrogen seems prevent this event, however, the GSH and estrogen effects on microdamage-induced osteocyte apoptosis probably occur through different mechanisms.

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Mechanism-based modeling approach relating human gut microbial community to physiologically-relevant biomarkers

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The adult human gut houses a microbial community which contains a large number of bacterial species. It is well-known now that the actual composition of this community has a significant influence on human vital functions and may be an important determinant of various pathologies. However, the mechanisms controlling the assembly of gut microbiota and its relationship with host human tissues remain poorly understood. This paper represents a first attempt in developing an integrated quantitative description of literature-reported changes in butyrate, acetate and propionate in response to different bacterial composition (in accordance to the data published by Mahowald et al., 2009). It was shown that different steady-state ratios of short-chain fatty acids produced by one or another microbial composition can be considered as risk factors for obesity.

A mechanistic model of the relationship between human gut microbial community and host tissues was developed. The model can be used to evaluate the potential effect of various compositions of microbial community to the steady state ratios of short-chain fatty acids and in silico testing of possible therapies related to interventions and changes in gut microbial composition.

Spatially distributed models of evolution in symbiotic/antagonistic prokaryotic communities

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In nature, prokaryotes live in huge communities, evolution of which can not be reduced to the evolution of separate individuals or populations. One of the main tools for studying evolutionary mechanisms in such communities is mathematical modeling and computer simulations. Our software package ‘Haploid evolutionary constructor’ (HEC) allows modeling of genetic, metabolic, population and ecological levels of organization [1]. In HEC models, we consider both habitats with ideal-mixing of substrates and cells (0D), and spatially distributed habitats (1D, 2D and 3D).

The model of evolution of ‘poisoner-prey’ community consisting of two populations has been constructed. Population P1 poisons population P2 by S2 substrate, while P2 feeds P1 by S1 substrate. In this model (like in predator-prey model), one may observe various dynamics (steady states and oscillations). We studied the evolution of P1 and P2 in 0D model. In presence of genetic polymorphism in either P1 or P2 we observed directional selection: in P1 – maximization of S1 utilization efficiency; in P2 – minimization of S2 sensitivity. It is interesting that the rate of genetic diversity loss (i.e. removal of ‘weaker’ alleles) was almost twofold higher for prey population P2 (initial genetic diversities in P1 and P2 were the same). Addition of the spatial distribution to the model on the one hand, complexifies evolutionary behavior of the community; and on the other hand, makes it more resistant to mutations.
We have also studied some spatially distributed (1D-3D) models of genome complexity changes in symbiotic prokaryotic communities. Previously we had shown how habitat conditions may affect evolutionary trends in such communities (0D case); pessimum conditions promote genome amplification of communities, while comfortable conditions promote genome reduction [2]. We have shown that in case of 1D-3D models, genetic diversity changes (losses) in a community may significantly vary in different spatial regions, which encourages origin of non-trivial evolutionary scenarios.

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References

SW06.W30–33
Stochastic jump behavior in heterogeneous media
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Diffusion and transport inside cells or organelles takes place at a mix of spatial and temporal scales. Thus, many aspects determine molecular diffusion profiles. Given molecular motion can be restricted by a large number of factors, one cannot help but wonder: how do molecules reach their targets within such short amounts of time?

The keys to the riddle are facilitated diffusion and directed transport, and many experiments have already confirmed such long-standing theoretical conjectures. However, even if such types of motion are deemed ‘directed’, one should keep in mind they are still underpinned by stochasticity, and this has to be explicitly accounted for. Unfortunately, highly resolved stochastic spatial simulations in spatially complex domains can be prohibitively expensive. Thus, in many of these cases, accurate and representative simulations may even be unfeasible.

Here, we present our recent results in a simulation study, where we test several random walk algorithms aimed at representing stochastic directed motion inside cells for their efficiency and accuracy. This is done by the important realization that, in some cases, motion consisting of a mix of diffusion and directed transport can be approximated by Lévy jump processes. We provide mechanisms of how to capture this stochastic spatial motion information and how to relate it to Lévy processes.

SW06.W30–34
A selection criterion for patterns in reaction-diffusion systems
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Turing systems, and reaction-diffusion equations in general, have long been considered plausible pattern formation models, and they have been established as such in several chemical and biological systems. Due to their direct applicability in several biochemical systems, much effort has been given to determine whether specific parameters and/or nonlinearities lead to predefined patterns. Many times, such question has been addressed heuristically, or by running large numbers of simulations of specific models.

Here, we will consider our recent study where we consider a reaction diffusion system on a planar domain and provide an analytic criterion for determining whether spots or stripes will be formed. In more complex settings, we verify our criterion numerically. Most importantly, our criterion is not tied to a specific model, and can be used for different systems of reaction-diffusion equations. We show its applicability in estimating parameters or predicting patterns from the Gierer-Meinhardt model and FitzHugh-Nagumo equations. Nevertheless, our methodology can be used to study general morphogenetic systems.

SW06.W30–35
Reduction of chemical reaction networks with distributed delays
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One of the fundamental goals of Systems Biology is to understand complex interactions between components of biological systems. At the cellular level, such interactions give rise to specific biological functions such as gene expression, molecular transport and cell signal transduction, and are typically represented by chemical reaction networks. However, even when biological functions have been studied in scrutinizing detail and can be reliably represented by sets of chemical reactions, one may not be able to accurately simulate such networks, nor explore alternatives to wild-type scenarios. The reason for this is the associated computational costs, limiting the time spans in which phenomena can be simulated. Even when solely considering deterministic scenarios, the network of interactions can be very large, making the simulation of a system potentially unfeasible. Thus, there is a great need to reduce networks of chemical reactions.

Recently, we propose a methodology aimed at representing chains of chemical reactions by much simpler, reduced models (Barrio et al., J Chem Phys (138) 104114, 2013). The abridgement is achieved by generation of model-specific delay distribution functions, consecutively fed to a delay stochastic simulation algorithm. We show how such delay distributions can be analytically described whenever the system is solely composed of consecutive first-order reactions, with or without additional ‘backward’ bypass reactions, yielding an exact reduction. For models including other types of monomolecular reactions (constitutive synthesis, degradation, or ‘forward’ bypass reactions), we discuss why one must adopt a numerical approach for its accurate stochastic representation, and propose two alternatives for this. In these cases, the accuracy depends on the respective numerical sample size. Our model reduction methodology yields significantly lower computational costs while retaining accuracy. Quite naturally, computational costs increase alongside network size and separation of time scales. Thus, we expect our model reduction methodologies to significantly decrease computational costs in these instances. We anticipate the use of delays in model reduction will greatly alleviate some of the current restrictions in simulating large sets of chemical reactions, largely applicable in pharmaceutical and biological research.
MicroRNA determines the early stage dynamics of the regulation network

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One of the regulatory mechanisms of gene expression extensively studied in the last years involves miRNAs. It is realized now that this function of miRNA can be explained only via understanding its role in the regulatory network comprising miRNA interacting with transcription factors (TFs). One of the modern interaction models was introduced in [1] in the form of the coupled kinetic o.d.e. and solved at the steady state approximation \((\dot{c} = 0)\) in both deterministic and stochastic statements. The transcription rates of the miRNA gene and of the target gene were assumed to be the Hill functions of the number of TFs, while the translation rate of the target gene – a repressive Hill function of the number of miRNAs. Evidently, one and the same stationary solution to the system can fit to solutions, which are completely different at the early stage of development, and some of them may not even have any biological sense, that cannot be verified in the algebraic version of the o.d.e.

We obtain the exact solutions to the coupled o.d.e. under some biologically relevant restrictions and find the detailed dynamics of both coherent and incoherent networks. The remarkable variations of concentration at early stage were shown, invisible in the steady state solutions. We have found that depending on the initial conditions, several concentrations, being acceptable at steady state, may be negative at early stage, which is inconsistent with biology.

The exact solutions obtained allow us to describe the parameter ranges, within which the model behavior is able to suppress the noise occurring in the regulation loop. The solutions are useful to reconstruct the set of proper parameters to avoid meaningless concentration values at early stages in both deterministic and stochastic models.

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References


A phenomenological model of iron-mediated complications of aging diseases

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Main principles of General Systems Theory were applied to analyze purposeful relations between functional systems in damaged tissue. Among others, the system aimed to protect a tissue from iron-mediated damage was revealed as a critical one.

It is known that iron accumulates in tissues with age. This results from a pattern of systemic iron turnover which does not involve specific mechanisms of iron extrusion. Iron overload correlates with tissue damage and is suspected to result in complications of cancer, AS, COPD, diabetes, neurodegenerative diseases etc. A core mechanism responsible for iron-mediated oxidative tissue injury is a catalytic cycle of Fenton/Haber-Weiss reactions, a source of hydroxyl radicals. Iron overload which is enhanced in aging amplifies the oxidative damage.

The presented model illustrates a hypothesis: a disturbance of macrophage-mediated local iron turnover in damaged tissue results in increased probability of fatal complications of the disease. A key point of the disturbance of local iron turnover is local induction of expression of hepcidin by resident macrophages. This results in suppression of iron release from macrophages. Therefore, the macrophages engulf apoptotic and damaged tissue cells, process iron but are unable to recover via release of the accumulated iron locally. Thus resident tissue macrophages have to leave the tissue. As a result, the system responsible for tissue clearing and recover enlarges via involvement of additional sub-systems necessary for macrophage release, including temporary stroma rapture and regeneration, angiogenesis, lymphotoigenesis etc.

Physiological outcomes of the model: (i) early diagnostics of a switch of local damage into systemic one may help to prevent diseases development; (ii) gene polymorphism of the proteins involved in the extended system of macrophage-mediated iron turnover may give a basis for personal prediction of a probability of the diseases complications; (iii) intravenous iron injection should be applied with a caution even in anemia, unless liver iron store is depleted.
SW06.W30–39
Mode of murine hippocampal cell death after treatment with cationic phosphorus dendrimers
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Cationic phosphorus dendrimers (CPD) have been proved to possess an array of potential biomedical applications. Nevertheless, the positive charge they carry, render them relatively highly cytotoxic. The toxicity of CPD has been so far evaluated based on standard viability tests, while there is scarce information on their mechanism of action and influence on cell condition. Therefore, we decided to investigate processes occurring in cells (in vitro) in response to treatment with two generations (G) of CPD – G2 and G3. Due to increasing amount of works focusing on the ability of CPD to inhibit structural alterations of proteins involved in neurodegenerative disorders (Alzheimer’s and Parkinson’s diseases, prion infections), we chose new murine hippocampal cell line (mHippoE-18) as a model for our research. Dendrimers application to these cells at concentration above 0.7 and 1 μM for G2 and G3, respectively, led to pronounced reduction in cell viability, which correlated with massive production of reactive oxygen species (ROS), mitochondrial membrane potential (ΔΨm) dissipation and morphological changes. Only slight increase in the percentage of fragmented DNA and in cytosolic cytochrome C was observed. Moreover, no induction of caspase 3 activity was detected. These data indicate that CPD did not induce apoptosis in hippocampal cells, which died mainly through the necrotic pathway. Our findings are of high significance since necrosis, as a proinflammation process, is highly undesirable in the organism. Thus special care should be taken, while establishing proper dosages of CPD in future in vivo studies. Furthermore, the results suggest that designing of CPD grafted with compounds such as carbohydrates, polyethylene glycol, or acetyl groups, reducing their cytotoxicity would be a good direction of future investigations on CPD cytotoxicity.

SW06.W30–40
RAPD-PCR analysis of subgenus Terricola (Mammalia: Rodentia) in Turkey
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Subgenus Terricola have 14 species. Three of this species (Microtus majori, Microtus daghestanicus and Microtus subterraneus) were distributed in Turkey. In this study, Microtus majori (Thomas, 1906) and Microtus subterraneus (de Selys-Longchamps, 1836) species were studied. Microtus majori’s dispersal area is on the east black sea region of Anatolia in Turkey. Microtus subterraneus’ dispersal area is on the black sea region of Anatolia, western Anatolia and Thrace in Turkey. In previous studies have revealed that Thrace samples of Microtus subterraneus have 2n = 52 and Anatolia samples of Microtus subterraneus have 2n = 54. On both sides of the Bosporus, the survival of two different chromosomal forms belong to the same species is important, and genetic examination of these two different populations is of great importance. Aim of the study is to determine if any inter and intra specific variation in Microtus majori and Microtus subterraneus populations in Turkey using RAPD (Randomly Amplified Polymorphic DNA). In this study, we used 41 samples collected from 14 populations. With the 60 RAPD markers tested, 10 of them yielded 167 polymorphic DNA bands. All estimations were calculated with the POPGNE and TFFPGA software. Result of these calculations, the estimated genetic distance according to Nei (1972) was ranged from (D) 0.0258 [Microtus subterraneus (2n = 54) – Microtus majori (2n = 54)] to (D) 0.1355 [Microtus subterraneus (2n = 54) – Microtus subterraneus (2n = 52)]. According to dendogram constructed based on Nei’s genetic distance, Thrace populations were separated from Anatolian populations.

SW06.W30–41
The effect of fullerenol C_{60}(OH)_{36} on human erythrocyte morphology and acetylcholinesterase activity
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Fullerenes have high electron affinity, therefore they tend to bind free radicals and nucleophiles. Moreover, the results of many studies prove the non-toxicity of water-soluble, hydroxyl derivatives of fullerenol C_{60} – fullerenols C_{60}(OH)_{n}. However, there are a few studies regarding the toxic effects of fullerenol.

We have studied the effect of fullerenol C_{60}(OH)_{36} on human erythrocyte morphology and acetylcholine esterase activity to assess its toxicity.

Human erythrocytes (hematocrit of 2%) suspended in PBS were incubated with FulOH (C_{60}(OH)_{36} + 16H_{2}O) at concentration range 0–150 mg/l at 37°C for 3 h in the dark. The changes in erythrocyte size and shape were assessed on the basis of light dispersion parameters FSC-A and SSC-A obtained by flow cytometry using a Becton-Dickinson, LSR II flow cytometer and observed using a phase contrast microscope (Eclipse E600W Nikon, Japan). In order to estimate the influence of fullerenol on the erythrocyte membrane integrity the osmotic fragility test was done. The activity of AChE was determined using the spectrophotometric method of Ellman et al. (1961). Acetylcholinesterase, the enzyme anchored in the outer layer of erythrocyte membrane, is a good tool for determining influence of many factors on the structural and functional activity of membrane components.

The results obtained in this study reveal a little impact of fullerenol on the erythrocyte morphology. The small changes in the morphology were observed at high concentrations of fullerenol, higher than 100 mg/l. The SSC-A histograms provide information about the shape and structure of the outer surface of the plasma membrane. After analyzing the percentage of unaffected cells it was visible that this parameter slightly decreased (7%) only in the samples treated with fullerenol at the concentration of 150 mg/l. These changes did not lead to the increased red blood cells permeability. Moreover, the AChE activity did not change under the influence of fullerenol up to 150 mg/ml. Fullerenol did not exhibit antiesterase properties although reversibly increased membrane fluidity and irreversibly inhibited the ATPases responsible for enabling asymmetric concentrations of cations across the membrane which was proved in our previous paper.
Platelet activation by endocannabinoids

**SW06.W30–42**

**Coupling bioinformatics and experimental approaches to elucidate the role of orphan human proteins**

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Several thousand human proteins are still uncharacterized and cannot be assigned an activity by comparison with proteins of known function. The CALIPHO group (Computer Analysis and Laboratory Investigation of Proteins of Human Origin) applies bioinformatics methods (e.g. data mining in available data sets, sequence and phylogenetic analysis) to build functional hypotheses for some of these orphan proteins, and validates them using human cell lines and/or Zebrafish as models.

We have demonstrated the role of APIP in the methionine salvage pathway (Mary et al., 2012) and the role of DERA in deoxyribose recycling (Salleron et al., in preparation), and are presently focusing on orphan mitochondrial proteins that could be involved in metabolic diseases and on orphan proteins involved in clonogenic and/or chemoresistance.

We are eager to collaborate and share our bioinformatics expertise with any laboratory interested in the characterization of human orphan proteins.

**Reference**


**SW06.W30–43**

**Platelet activation by endocannabinoids through the arachidonic acid pathway and PPARgamma involvement**

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Endocannabinoids are lipid signaling molecules involved in a variety of physiological and pathological conditions. The two main endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (N-arachidonylethanolamine, AEA) are synthesized by neurons and other cells, including platelets, in a stimulus-dependent manner. Following their transport into cells they are inactivated by enzymatic degradation. AEA is inactivated by fatty acid amide hydrolase (FAAH), whereas 2-AG is inactivated by the action of both FAAH and monoacylglycerol lipase (MAGL). In this study, the degradation process of 2-AG by FAAH and MAGL and the characterization and identification of the enzymes in rabbit platelets using immunoblot analysis and immunochemistry is reported. We suggest that platelets are activated by the endocannabinoid 2-AG via its hydrolysis to arachidonic acid. Preincubation of platelets with FAAH and MAGL inhibitors affected the aggregation induced by 2-AG and other platelet agonists (PAF, thrombin). Regarding human platelets, reports are controversial; the activation of platelets by endocannabinoids through the activation of cannabinoid or ‘platelet type’ receptors has been reported while other reports, including results from our group suggest that platelets are activated, at least in part, by the produced arachidonic acid. Finally, our finding regarding the presence (and alterations) of PPARgamma in platelets, suggest a role for this nuclear factor in platelet activation induced by endocannabinoids. The functional role of endocannabinoids in platelets is not clear. It appears though that these lipids play a major role in the arachidonic acid homeostasis and the consequent effects on platelet activation and suggest that platelets contribute to the inactivation of the endocannabinoids.

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**SW06.W30–44**

**Study of a protein function of KCTD-family**

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Studying the causes of B-cell chronic lymphocytic leukemia, we found several protein-coding genes. One of the interesting candidates for the role of tumor suppressor gene was KCNRG. Bioinformatic analysis showed that its amino-acid sequence contains the T1 domain responsible for protein-protein interactions through tetramerization. Genome search revealed 27 other human genes with T1-domain, which groups them into a family of KCTD-proteins.

We have hypothesized that the KCNRG disrupts the assembly of tetramers of potassium channels, also possessing the T1 domain. We have shown that the KCNRG protein inhibits voltage-dependent potassium currents in cells of prostate carcinoma LnCaP, and stable over-expression of the gene KCNRG leads to suppression of proliferation and increased apoptosis in model cell lines of prostate carcinoma LnCaP, multiple myeloma RPMI-8226 and in promyelocytic leukemia HL-60 cells. This proves that the KCNRG gene has tumor suppressor properties.

Despite the high degree of similarity with other KCNRG-like proteins, KCTD5 protein in solution does not form tetramers, but pentamers, whereas the tetramers are very unstable. Interestingly, the KCTD5 protein actively interacts with other proteins (non-potassium channels) through its C-terminal module, as well as through other epitopes. For the rest of the members of this family information about their interaction is scarce.

We carried out the analysis of literature on KCNRG-like proteins and analyzed the participation of each unique member of the KCTD-family proteins in various signaling cascades and cell systems. In functional terms, family of KCTD proteins is quite diverse.

In order to understand the function of KCNRG-like proteins and their interaction with potassium channels, we use bicistronic expression system for simultaneous expression of one of target KCTD gene and potassium channel. Also, we investigated basic cell function of some interesting KCTD genes for analysis it’s proliferation and apoptosis properties.

**SW06.W30–45**

**The prooxidative effect of resveratrol in a neuroblastoma cells**

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Neuroblastoma is a kind of cancer which occurs mostly in infants and young children. Often these malignant tumors are resistant to chemotherapeutic drugs. For these reasons, more effective treat-
ments are needed. Resveratrol (3,4,5′-trihydroxy-trans-stilbene), a natural polyphenol, has been shown to exert anticancer effects in different systems based on its ability to inhibit diverse cellular events associated with tumor initiation, promotion, and progression. Resveratrol inhibits the growth of several cancer cell lines, which suggests that it also has an inhibitory effect on cancer progression.

In this study, we investigated the effect of resveratrol on neuroblastoma cells (Neuro-2a) treated with hydrogen peroxide or nitric oxide-releasing compound, NOC-18.

After 24 h incubation of Neuro-2a cells with H2O2 or NO respectively reduced the viability of the cells in dose-dependent manner. The concentration of the mentioned oxidants which caused 50% reduction in cell viability was 0.02 mM and 0.25 mM for H2O2 and NOC-18, respectively. Cells treated with resveratrol 3, 6 or 12 h prior to H2O2 or NOC-18 treatment exhibited significant reduction in survival compared to cells treated with hydrogen peroxide or nitric oxide alone. After resveratrol treatment, the apoptosis of the neuroblastoma cells exposed to hydrogen peroxide or NOC-18 increased significantly.

In conclusion, resveratrol in combination with hydrogen peroxide or nitric oxide, increases cellular cytotoxicity and inhibits the proliferation of neuroblastoma cells.

**SW06.W30–46**

**Interaction of Mycoplasma gallisepticum with host-cell organelles**

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The genus *Mycoplasma* relates to Gram-positive bacteria that lack a cell wall and are capable to cause chronic disease in humans and animals. The capability to affect the development of apoptosis in the host cells was shown for a number of mycoplasmas.

The object of the present study is a Mollicutes class bacteria *Mycoplasma gallisepticum* owning parasitic life style in poultry. A number of studies show that *M. gallisepticum* is capable to penetrate into eukaryotic cells. The ability of *M. gallisepticum* to persist in the host, as well as its participation in the development of apoptosis suggests that it could affect the host cell’s and intracellular organelles’ metabolism. In particular, possible cooperation between mycoplasma and mitochondria and especially their effect on the initiation of the mitochondrial apoptosis pathway is exiting.

We analyzed the interaction of *M. gallisepticum* with mitochondria isolated from Gallus domesticus liver tissue using confocal laser scanning microscopy (CLSM) with fluorescent staining. We demonstrate that *M. gallisepticum* is able to adhere to the mitochondrial surface. Trypsin treatment abolishes such interactions, which may indicate its protein-protein nature. However, we show that other species of the Mollicute class (*Acholeplasma laidlawii*, *Spiroplasma melliferum*) do not have such ability to interact with mitochondria. Interestingly, we did not observe interaction of *M. gallisepticum* with other intracellular organelles (for example, chloroplasts isolated from *Pisum sativum*).

We suggest that Mycoplasma – mitochondria interaction can affect the functional activity of mitochondria in the host cell and also play role in the triggering of the mitochondrial apoptosis pathway.

**SW06.W30–47**

**Induction of cell responses to the endogenous expression of Bacteroides fragilis toxin in culture HEK-293**

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The development of high-tech sequencing of nucleic acids resulted in the start of human metagenome projects. The microbial communities composition colonizing the large intestine, led to the creating a database of bacterial genomes. Despite the variety of learning mechanisms of bacterial and epithelial cell interaction, the role of some bacterial toxins is unknown. Remains uncertain role of toxins, that are freely enter to epithelium cells. The research purpose was in the creation and study of intracellular induction of cell responses to the endogenous expression of *Bacteroides fragilis* toxin (BFT) in the cell line HEK-293. To identify the BFT protein produced in cell culture as a result of gene expression Western blot has been used. In our experiments it was shown, that only immature inactive form of toxin is produced in epithelium cells line HEK-293. However, the intracellular effects of the toxin have identified a number of quantitative changes in the protein level and RNA transcription. We demonstrate the absence of the mature form of toxin that is expected as a result of the BFT protein processing. Although, we detected high levels of toxin mRNA in HEK-293. This suggests either, rapid degradation of processed BFT protein or lack of the normal processing. This effect may indicate that host cell is altered by the plasmid encoding BFT or by the transcribed BFT RNA and that such alteration promote the described changes of host cell translation and transcription.

**SW06.W30–48**

**The role of nitric oxide in testicular sperm extraction (TESE)**

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Many conditions or events associated with male infertility are inducers of oxidative stress. Such stress conditions can cause changes in the dynamics of testicular microvascular blood flow, endothelium signaling, and germ cell apoptosis. Testicular oxidative stress appears to be a common feature in much of what underlies male infertility. In recent years, scientists have studied the role of nitric oxide (NO) in the male infertility. There are different physiological roles for NO in male reproductive system. NO is a potent vasodilator and cell signaling molecule can play its own role in amplifying testicular injury. The aim of study is to evaluate NO and oxidative stress in testicular tissue in infertile men with azoospermia cases either do not have spermatozoa or have any spermatozoa. In this study, testicular biopsies were obtained from 20 men with azoospermia who were attended to infertility center for diagnosis or infertility treatment. TESE samples were divided to two groups as spermatozoa were detected and not detected for azoospermic men. Immunohistochemistry was used to localize the all three of nitric oxide synthase (NOS) isoforms in these tissues. Chemiluminescence
measurement of NO is based on the reaction of hydrogen peroxide and NO to peroxynitrite. Endothelial NOS (eNOS) reaction in spermatozoa detected group was considerably higher than spermatozoa absent group which shows that eNOS plays an important role in spermatogenesis detection. Inducible NOS (iNOS) reaction was also higher in comparison to eNOS reaction. It was observed that iNOS reaction was higher than spermatozoa absent groups. There was no significant difference in the neuronal NOS (nNOS) reaction between the spermatozoa detected and spermatozoa absent groups. Superoxide radical generation in spermatozoa detected group was significantly lower than spermatozoa absent group. Peroxynitrite ratio in spermatozoa absent group was significantly higher than spermatozoa detected group. These results were showed that three isoforms of eNOS and iNOS play an important role in spermatogenesis process in azoospermic men. However; nNOS may act as a signal molecule for spermatogenesis process. In conclusion, testicular oxidative stress plays a role in male infertility.

**SW06.W30–49**

Out-of-peak ChIP-seq signal analysis and approach to ChIP-seq peaks and protein–protein interaction usage for protein complex reconstruction

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ChIP-seq is very important method for protein-DNA interaction examination. Usually it is used for examination of relatively constant protein-DNA binding that is represented by peak in signal but out-of-peak signal seems to be not used. In this work out-of-peak signal analysis and its applications are described. Integrative approach for ChIP-seq peaks and protein-protein interaction usage is described as well.

Out-of-peak ChIP-seq signals were derived from general ChIP-seq signal by annotated peaks exclusion. UCSC Browser was used as a source of ChIP-seq signals. ChIP-seq signals for different transcription factors and RNA polymerase II were used. Firstly it occurred that out-of-peak signal can be used for chromatin remodeling complex and general chromatin events reconstruction. One of applications was RNA polymerase II elongation complex. It occurred that out-of-peak signal is higher in genes and especially in exons than out of genes and exons respectively. Moreover out-of-peak signals of RNA polymerase II and its transcription factors were positively correlated but RNA polymerase II out-of-peak signal was not positively correlated with RNA polymerase III transcription factors. Original controls were used and strong underestimation of genes regions in genome was taken into account. According to results new model for elongation complex was suggested were RNA polymerase II was bound to transcription factors during elongation process. Model where it stays connected to promoter through transcription factors is also discussed.

ChIP-seq peaks were used for database approach where it was used along with protein-protein interactions. Many potential protein complexes were reconstructed. Those complexes included histone modifiers and chromatin remodeling complexes. This information can be used in docking for better understanding of orientation of proteins subunits in complexes.

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**SW06.W30–50**

Rule-based model of bacterial transcription initiation

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RNA-polymerase of *E. coli* is able to recognize more than 7000 promoter areas on the bacterial chromosome (Salgado, 2012). The key missing part in current understanding of the mechanism of transcription regulation is the mechanistic understanding of the process of promoter location by RNAP and its consequences for the promoter efficiency. It was estimated that the number of RNA-polymerase (RNAP) molecules vary between 1500 and 11 000 per bacterial cell (Klumpp, 2008). It was also shown that about 80% of RNAP are not engaged in transcription process as they are bound to the chromosome in non-specific manner. The subunit required by RNAP for promoter recognition and transcription initiation, σ-subunit, even more rare: there are 500 σ70, 95 σ32 and 55 σ34 molecules per cell (Ishihama, 2000). Each promoter has slightly different sequence and as the consequence, RNAP interacts differently with each promoter.

We have developed *E. coli* transcription initiation model with the rule-based approach (Danos, 2009). The key feature of this model is the presence of non-specific DNA and rules that account for difference in interaction of RNAP with promoter and non-promoter DNA.

To make simulation setup similar to the *in vivo* environment we have developed new analysis technique called concurrent sensitivity analysis. Global sensitivity analysis probes behavior of the model by its simulation with a number of parameter sets evenly covering some hypercube in the parameter space. Usually such simulations are performed similar to *in vitro* experiment: model is simulated with each parameter set like each promoter tested in its own test tube. Parallel setup is. A rule-based simulation allows us to combine submodified models with all parameter sets into one model in a concurrent environment, which more reminding *in vivo* experiment.

The global sensitivity analysis of model simulations in concurrent shows that signs and values of sensitivity coefficients are similar in both *in vivo* and *in vitro* setups. What is interesting on that graph is that non-specific binding coefficients has sensitivity close to parameters of ‘open’ complex formation, which are known to be the key parameters in the transcription initiation model.

That results emphasize that in concurrent environment where some molecules are in deficit, weak non-specific interactions could be more important then strong interactions, which found significant during *in vitro* experiments.

**References**

SW06.W30–51
Automatic transcription regulation model creation with kGraphProm

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E. coli chromosome believed to encode about 8547 promoters [1], so it contains about one promoter per 700 bp. There is also about two hundred transcription factors controlling activity of that promoters via 2155 binding sites [1]. It is also known that 56% TGs are regulated by more than one TF in E. coli [2]. Dynamical modelling of system of that complexity is difficult task. To deal with that kind of complexity we need a tool to make a model of smaller size that contains all elements required for description of particular subsystem behaviour. We have developed software pipeline kGraphProm that provide such functionality.

The kGraphProm consists of thee parts: (1) graph database stores interactions between promoters, genes and transcription factors; (2) database of the submodel templates describing the specific details of regulation of the particular interaction; and (3) model builder to combine individual submodels into one model.

References

SW06.W30–52
Uranium bioleaching – insight into the structure of microbial consortia from mining tailings

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Bacterial ore leaching can be applied to extract heavy metals from low grade ores or industrial wastes. Numerous mine spoils, slag dumps and tailings remained in Silesia (SW Poland) area of former uranium exploitation. The differential content of U reaches up to 0.02%.

Six taxonomically stable consortia have been isolated from different locations of the heaps containing relatively high content of uranium. They were cultured on two minimal media (Beijerinck’s and 9K) promoting growth of sulfur and ferrous oxidizing bacteria in aerobic conditions. Total DNAs of consortia were pyrosequenced (using 454 FLX Titanium Roche technology), assembled and taxonomic and functional assignments performed using MEGAN software. The consortia were distinct from phenotypic point of view; therefore, we merged the samples cultured on the same media for the purpose of the analysis of biodiversity, but kept separate for the purpose of functional analysis. Here we are showing that media with two different energy sources lead to dramatic differences in the structures of the consortia, yet the diversity of both is still relatively high. The only exceptions are genus Acidithiobacillus, Acidiphilium and Leptospirillum, which are present in the communities cultured on both media. The presence of Fusariun species in Beijerinck’s cultivated consortia was noted.

Enzymes specific to the particular energy source were present but not in a large number, confirming that survival of the consortium depends on a few species. We postulate that the presence of obligatory chemolithoautotrophs makes the microbial consortia in these oligotrophic environments resistant to changes in the availability of energy sources.

SW06.W30–53
Study of the kinetics of transcription as a function of the cell growth phase

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Gene expression is mainly controlled during transcription initiation, a process whose kinetics has been hypothesized to be growth phase-dependent. Using time-lapse microscopy, we study with single-molecule sensitivity the growth phase-dependent kinetics of mRNA production when under the control of P(lac/ara-1) and of PBAD.

We use MS2-GFP, an RNA-tagging protein, to detect individual mRNA molecules, as these are produced. Each cell contains a single copy BAC vector with a target promoter, followed by a region coding for 96 binding sites for the MS2d coat protein. A medium-copy plasmid encodes for MS2d-GFP and is under the control of P(lac/ara-1)-Target RNA molecules can be observed as fluorescent spots.

Cells containing the plasmids were treated with l-Arabinose and IPTG, to activate the target gene, and with aTc, to activate the reporter gene. Images of the cells were acquired by a confocal microscope, to detect the spots in the cells. From the images, using image analysis, we extract the number of target RNA molecules produced by each cell, the mean and standard deviation of number of RNA molecules per generation, and the intervals between subsequent transcription events in each cell. From the latter, we infer the number and duration of rate-limiting steps in transcription. Also, we assess the noise of the process of transcripts production at each growth phase.

SW06.W30–54
Examination of angiopoetin-like protein 4, neuropeptide Y, Omentin-1 levels of obese and nonobese patients with polycystic ovary syndrome

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The pathogenesis of PCOS and obesity is not clarified yet. But some parameters such as NPY, and Angptl-4, Omentin-1 are thought to be involved in this pathogenesis. In this study, we aimed to show possible effect of these parameters. Seventeen obese, 32 non-obese patients with PCOS and 20 healthy subjects were included in this study. The control group was consisted of healthy patients, who had and having no PCOS or DM history. PCOS and obesity were diagnosed in Kirikkale University Faculty of Medicine. The demographic parameters of all patients were analyzed in Biochemistry Laboratory of Kirikkale University Faculty of Medicine. Levels of serum NPY, Omentin-1, Angptl-4 were measured at Gazi University School of Medicine, Department of Medical Biochemistry. Patients were divided into three groups. Group I was consisted of 20 healthy volunteers, 32 non-obese women with PCOS was named as group II and group III was consisted of 17 obese women with PCOS. Serum NPY, Angptl-4, free testosterone, total testosterone, LH, SHBG, estradiol, DHEA-SO4, androstenedione, TG and LDL-C levels and HOMA-IR, Ferriman-Galwey scores were significantly higher in group II when compared group I and similarly in group III comparing with group II (p < 0.005). When comparing all PCOS patients (obese + non-obese) with healthy volunteers; omentin-1 and HDL-C levels were significantly low in PCOS group (p < 0.005). In obese PCOS patients NPY, Angptl-4, HOMA-IR, BMI, and waist circumference levels were significantly higher,
while Omentin-1 levels lower than those in non-obese PCOS patients (p < 0.005 for both of them). As a result of this study, both in the obese and non-obese PCOS patients, there was a significant increase in levels of NPY and Angptl-4 and a significant decline in the anorexigenic peptide, Omentin-1 when compared to healthy subjects. In conclusion, insulin resistance in PCOS patients may be related to the differences of NPY, Angptl-4 and omentin-1 levels and the effects of these differences on metabolic pathways.

**SW06.W30–55**

Transcription regulation in prokaryotes: the role of electrostatics as a natural selection factor. DNA phenotype and biophysical bioinformatics

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Genome DNA physical properties (PP) influence its interactions with proteins, esp. regulating transcription. DNA is highly charged and electrostatics contributes greatly to the subject. However, lack of methods to calculate electrostatic potential profile (EP) of long DNA stretches prevents the problem researches. Such method had been developed in our laboratory. Here we built DEPPDB – database of electrostatic and other PP of all complete sequenced genomes, bound to taxonomy and genomes annotations.

We found that negative EP has a nonuniform distribution along DNA molecule and correlates with the GC content, though does not correspond to it exactly, and strongly depends on both the sequence arrangement and its context. Direct experimental binding frequency of RNA polymerase to DNA along the genome correlates to the calculated EP.

Transcription regulation areas have EP and other PP peculiarities. Binding sites of transcription factors lie in high EP areas and have high EP, corresponding to that of TF proteins. Promoters have high value and heterogeneity of EP profile. Transcription starting sites of prokaryotic genomes are surrounded by hundreds bp of high EP and peculiarities around TSS, similar in related taxa, due to protein binding and formation of other EP, needed for transcription machinery. Promoter up-element seem to have electrostatic nature.

Evidences exist of EP and other PP interactions in their formation and in transcription regulation.

The data present the importance and universality of the role of electrostatics in transcription regulation of prokaryotic genomes. The proposed mechanism affects the binding probability and positioning accuracy of proteins involved in transcription regulation. The universal nature of the regulatory impact of electrostatics suggests its importance to the process of horizontal gene transfer and the evolution of transcription regulation systems and contributes to understanding of high AT content of genome regulatory regions.

Physical properties formation affect fundamental problems: Chargaff's II rule, genetic code redundancy, synonymous substitutions neutrality. They justify fundamental idea of DNA phenotype, and define a new principle of biophysical bioinformatics.

DEPPDB is available at http://deppdb.psn.ru

**SW06.W30–56**

Variable patterning in Drosophila embryos due to basins of attraction in underlying gene regulatory dynamics

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Fruit flies are model organisms for studying spatial pattern formation in animals. In the first few hours of development, a network of interacting genes forms expression patterns which determine the body plan (chemical blueprint to guide morphogenesis). It is well established that wild-type (WT) development is remarkably robust, showing canalization of various initial trajectories to an attracting state [Surkova et al., 2008]. We work here with a gene circuit model of four genes – (hunchback; Kruppel, Kr; giant & knirps), under the control of the maternal Bicoid (Bed) and Caudal (Cad) gradients. The 4-gene model provides a small very well characterized network for investigating this. Dynamical systems analysis of the core model of the segmentation gene network has shown how this WT stability can arise [Manu et al., 2009ab]. The WT is stable only to a certain point, however [Gursky et al., 2011; Spirov & Holloway, 2012]. Strong gene mutations (deletions, insertions) can cause major (lethal) disruptions in the body plan.

Mutant patterns can be understood through bifurcations to pathological, non-WT, basins of attraction in a multi-stable phase space. Our work focuses on using weaker perturbations of genes (weak alleles) to more continuously move the gene network from the WT trajectory. (Particularly we deal with the weak alleles of Kr.) These gene variations can produce variable expressivity, in which the outcomes of a sample of embryos is not deterministic, but scatter between a selection of pathological outcomes (from nearly WT to strongly altered). We have shown how mutation of gene-gene interactions (particularly Cad protein effect on Kr gene) can lead a gene network to a bifurcation point, at which natural variability of the Bcd gradient can push embryos into neighboring basins of attraction. Our work suggests a dynamical basis, in which the variable outcomes are a manifestation of natural variability in upstream control. It can explain how mutations decrease the robustness of gene networks to natural variability. Understanding the model components and parameters which produce the experimental pattern perturbations allows us to create more detailed understanding of the biological regulatory dynamics used in body formation.

**SW06.W31 Biogenic Polyamines in Cell Metabolism (VI-W31)**

**SW06.W31–1**

The role(s) of polyamines in mammalian physiology

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Recent genetic, biochemical and studies have greatly clarified the essential functions of polyamines in rodents, microorganisms and parasitic protozoa. This work not only provides critical knowledge of cellular physiology but also reveals both the potential value of inhibitors or antagonists of polyamines as drugs and the care that should be taken to design such drugs to avoid toxicity. Direct evidence of the essential role of polyamines in normal growth and development of humans comes from studies of Snyder-Robinson syndrome, an X-linked recessive human disorder which results from mutations in the gene encoding spermine syn-
thase. Affected males have intellectual disability, hypotonia, ataxic body build, bone abnormalities, facial dysmorphism, ambulation difficulties, speech abnormalities, a high, narrow or cleft palate, short stature and a propensity to seizures. Genital and renal complications also occur in some individuals. Cells derived from these patients have only modest reductions in spermine but a large increase in spermidine suggesting that compensatory mechanisms attempt unsuccessfully to provide adequate polyamines. The presentation will discuss these changes in terms of the known functions, transport and metabolism of polyamines and the phenotype presented by Gy mice, which have an X-chromosomal deletion including the spermine synthase gene. These mice lack all spermine and have a greatly reduced size, sterility, deafness, neurological abnormalities, and a tendency to sudden death and poor bone development.

SW06.W31–2
Mechanism of polyamine stimulation of protein synthesis in eukaryotes
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Polyamines (putrescine, spermidine and spermine) are present at millimolar concentrations in eukaryotic cells and play regulatory roles in cell growth. Since polyamines exist mostly as polyamine-RNA complexes, we have extensively studied the proteins in Escherichia coli whose synthesis is enhanced by polyamines, and proposed that a set of genes whose expression is enhanced by polyamines at the level of translation can be classified as a ‘polyamine modulon’.

In eukaryotes, synthesis of Cct2 protein (T-complex protein 1, β-subunit) in FM3A and NIH3T3 cells and that of Cox4 protein (a subunit of cytochrome C oxidase, complex IV) in yeast were enhanced by polyamines through stimulation of the ribosome shunting of the stem-loop structure (hairpin structure) during the scanning of the 5′-untranslated region of Cct2 and Cox4 mRNAs by 40S ribosomal subunit-Met-tRNA complex. Furthermore, synthesis of eEF1A in FM3A and NIH3T3 cells was enhanced 3.0-fold by polyamines at the level of translation. It is thought that there is no SD (Shine-Dalgarno) sequence in eukaryotic mRNAs which exhibit complementarity to the nucleotide sequences at the 3′-end of 18S rRNA. However, complementary sequences consisting of more than five nucleotides to the 3′-end of 18S rRNA, eukaryotic SD (eSD), are present at −17 to −32 upstream from the initiation codon AUG in almost all kinds of mRNAs. In this case, proteins are synthesized with an SD sequence located at the 3′-end of 18S rRNA, eukaryotic SD (eSD). However, complementary sequences consisting of more than five nucleotides to the 3′-end of 18S rRNA, eukaryotic SD (eSD), are present at −17 to −32 upstream from the initiation codon AUG in almost all kinds of mRNAs. In this case, proteins are synthesized efficiently in the presence of polyamines increased 3.5-fold. The results indicate that polyamines stimulated eEF1A synthesis through the structural change of the eSD sequence located at the distant position from the AUG.

Reference

SW06.W31–3
The diversity of polyamine biosynthesis and function in bacteria
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The triamine spermidine is essential in all eukaryotes and variation in triamine biosynthesis is almost entirely at the level of whether spermidine is synthesized or whether the organism is dependent on exogenous spermidine. There is also variation in whether spermine and/or thermospermine is synthesized, with nearly all metazoans and angiosperm plants synthesizing spermine. Most protists do not synthesize spermine or thermospermine. In almost all cases in eukaryotes, spermidine, spermine and thermospermine are synthesized by a pathway using decarboxylated S-adenosylmethionine to provide the aminopropyl unit. The one known essential function of spermidine in eukaryotes is to provide the aminopropyl group required for the hypusine modification of the translation factor eIF5A.

In contrast, bacteria not only have alternative pathways for synthesizing spermidine, they may also synthesize alternative triamines syn-norspermidine and sym-homospermidine, both of which can be synthesized by alternative pathways. As with eukaryotes, some bacteria synthesize spermidine using decarboxylated S-adenosylmethionine and the aminopropyltransferase spermidine synthase. There is evidence that in some thermophiles sym-norspermidine may be synthesized by the same pathway. In many bacteria, e.g., most of the human gut microbiota, spermidine is synthesized from putrescine using aspartate beta-semialdehyde to produce a carboxyspermidine intermediate. This pathway is also used to produce sym-norspermidine from 1,3-diaminopropane in Vibrio species. Two completely different pathways are used to produce sym-homospermidine: one pathway uses the enzyme homospermidine synthase, an enzyme related to carboxyspermidine dehydrogenase and aspartate dehydrogenase; the other pathway uses a deoxyhypusine synthase-like enzyme. Different species within the same phylum may use different pathways to produce the same triamine, others use the same pathway to produce different triamines. More than one pathway may be present in the same strain and two different triamines can be produced in the same cell, especially spermidine and sym-homospermidine or sym-norspermidine and sym-norspermidine and spermine.

 Whereas spermidine is essential for growth and cell proliferation of eukaryotes, the role of polyamines in bacterial growth is less clear. In some species, e.g., Pseudomonas aeruginosa and Campylobacter jejuni, polyamines are critical for planktonic growth. In others, e.g., Escherichia coli and Vibrio cholerae, polyamine depletion slows but is not critical for growth. On the opposite side of the spectrum, spermidine is not required at all for optimal planktonic growth of Bacillus subtilis but is essential for robust biofilm development. Different polyamines are also essential for biofilm formation in the plague bacterium Yersinia pestis, and the cholera bacterium Vibrio cholerae.

SW06.W31–4
Polyamine synthesis and oxidation in the pathogenesis of immune dysregulation and gastric cancer caused by Helicobacter pylori
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H. pylori is a Gram-negative bacterium that selectively colonizes the human stomach and causes gastritis, peptic ulcer disease, and...
gastrointestinal cancer, the second leading cause of cancer death worldwide. Our laboratory has directly implicated polyamines in the persistence of the bacterium, ineffective inflammatory response, and development of cancer. We have reported that expression of ornithine decarboxylase (ODC), the rate-limiting enzyme for polyamine synthesis, is upregulated in macrophages in vitro and in vivo by \(H. pylori\) infection, and knockdown of ODC prevents induction of apoptosis and enhances antimicrobial nitric oxide (NO) production by enhancing translation of inducible NO synthase. We have implicated the polyamine spermine in the aberrant immune responses. Moreover, inhibition of ODC with chronic DFMO administration in the drinking water leads to reduced gastritis and \(H. pylori\) colonization levels in mice. In ODC\(^{-/-}\) mice there is also attenuated gastritis and colonization, and reduced Th1 and Th17 adaptive immune responses, an effect driven by enhanced TGF-beta and NO production in macrophages in the heterozygous mice. In gastric epithelial cells, induction of spermine oxidase (SMO), which backconverts spermine to spermidine and generates \(\text{H}_2\text{O}_2\), results in both apoptosis and DNA damage. Using flow cytometry, we have identified a subpopulation of cells in the infected stomach of mice and gerbils that simultaneously have high levels of SMO and DNA damage, but are resistant to apoptosis. We have implicated phosphorylation of epidermal growth factor receptor (pEGFR) in the survival of cells with DNA damage and in the induction of SMO in vitro and in infected mice treated with a pEGFR inhibitor. In gerbils, there is a marked reduction of \(H. pylori\)-induced dysplasia and carcinoma with inhibition of ODC or SMO, and an additive benefit with combination treatment. Studies in a human gastric tissue microarray indicate a strong correlation of SMO and DNA damage. Our phosphoproteomics and human tissue studies have implicated EGFR and ErbB2 signaling in addition to SMO in initiation of gastric carcinogenesis. Depletion of polyamines appears to eliminate deleterious effects of pEGFR in our models. Thus, ODC and SMO are key targets for prevention of gastric cancer.

**SW06.W31–5**

**New antitumor targets for polyamine-like compounds**

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Polyamine metabolism has been an antineoplastic drug target for over 30 years. Progress has been made in identifying the roles of polyamines in normal and neoplastic growth, aiding in the discovery of additional molecular processes that can be successfully targeted with specific polyamine analogues and drugs that interfere with polyamine metabolism or function. Recently, two discoveries have revealed new avenues for polyamine-like agents. The first is the recognition that infection/inflammation-induced polyamine catabolism is associated with carcinogenesis. Specifically, spermine oxidase (SMO), an FAD-dependent enzyme that catalyzes spermine to spermidine, 3-aminopropanal, and \(\text{H}_2\text{O}_2\), is rapidly induced by multiple mediators of inflammation and infectious agents associated with carcinogenesis, including gastric, colon, and prostate cancer. Induction of SMO produces oxidative DNA damage that can lead to mutations or epigenetic changes. We have demonstrated that an inhibitor of SMO blocks this damage and inhibits tumor formation. A second emerging target for polyamine-like compounds is the chromatin-remodeling enzyme, lysine-specific demethylase 1 (LSD1). LSD1 histone lysine demethylase is an FAD-dependent amine oxidase with high homology to SMO. LSD1 demethylates mono- and dimethyl-lysine 4 of histone 3, which are associated with transcriptionally active chromatin. LSD1 is sometimes associated with the aberrant silencing of tumor suppressor genes and is believed to be important in the etiology and progression of cancer. We hypothesized that LSD1 inhibition would induce the re-expression of aberrantly silenced genes and block tumor growth. Because LSD1 and SMO are structurally similar, a series of polyamine analogues were tested for their ability to inhibit LSD1. Several of these compounds are effective inhibitors of LSD1 and alone, or in combination with other epigenetic therapies, induce the re-expression of aberrantly silenced genes and inhibit tumor growth. These studies clearly indicate that LSD1 is a rational target for polyamine-like antitumor agents. Overall, these two targets for polyamine-like agents offer promising new avenues for both chemoprevention and chemotherapy. Strategies for exploiting these targets for therapeutic benefit will be discussed.

**SW06.W31–6**

**Inhibition of cellular proliferation and differentiation by polyamine depletion**

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The polyamines spermidine, spermine and their precursor putrescine are small organic polycations that are essential for the process of cell growth and proliferation. In most cases their depletion results in a reversible inhibition of cell proliferation. Polyamines were implicated with a variety of biological processes, however, which of these putative roles is essential for proliferation and viability is not presently known. Since it is impossible to selectively deplete polyamine supply to such individual processes, we have investigated the cellular consequences of global polyamine depletion. Our studies demonstrated that polyamine depletion resulted in strong inhibition of protein synthesis activity caused by inhibition of the initiation of this process due to phosphorylation of eIF2a. Biochemical analysis demonstrated differential activation of the PKR-like endoplasmic reticulum kinase (PERK) arm of the unfolded protein response and in parallel of the stress-induced p38 MAPK. These changes were accompanied by induction of key growth-inhibitory proteins such as p21 and Gadd45a and reduced expression of various cyclins, most profoundly cyclin D1, which even by itself can set the basis for the halted proliferation. However, although the induced stress response could arrest growth, polyamine depletion also inhibited proliferation of p-eIF2a and p-p38-deficient cells, suggesting that additional yet unidentified mechanisms might inhibit proliferation of polyamine-depleted cells. Indeed, our studies suggest that inhibition of mTOR activity reflected by reduced 4EBP phosphorylation is an alternative way by which polyamine depletion may provoke inhibition of protein synthesis activity and of cellular proliferation. Interestingly, we have recently revealed that some of the molecular players whose induction seems to be part of the mechanism by which polyamine depletion provokes proliferative arrest, also play a role in inhibition of some differentiation processes due to polyamine depletion.
In the autoregulatory network of the intracellular levels of polyamines, ornithine decarboxylase antizymes (AZs) and antizyme inhibitors (AZINs) are proteins that play a relevant role. AZs are polyamine sensors that negatively regulate polyamine biosynthesis and uptake, whereas AZINs act as positive regulators by inhibiting the action of AZs. The most recently identified antizyme inhibitor is AZIN2 [1]. In functional assays using transfected cells, AZIN2, as AZIN1, stimulates ornithine decarboxylase activity and polyamine uptake. AZIN2 is also like AZIN1, a monomer under physiological conditions that interacts with the three AZs. However, tissue and cellular distribution of both AZINs is different. Whereas AZIN1 is ubiquitously expressed, and in some cases it has been related with cell proliferation, early analysis of AZIN2 mRNA revealed its presence in terminal differentiated cells of brain and testis, where the protein appears to be associated with membranous structures of the Golgi network. In order to have more precise data on AZIN2 expression and obtain information of relevance for its possible physiological role, we have generated transgenic mice with a truncated AZin2 gene fused to the bacterial lacZ gene. Our results with these transgenic mice corroborate that AZIN2 is expressed in numerous brain areas and in haploid testicular cells but, interestingly, they show that this protein is also present in other tissues and cells, such as the pancreatic beta-cells and adrenal chromaffin cells. These and other results suggest that AZIN2 may have a role in differentiated cells, possibly affecting secretory processes through the regulation of intracellular polyamine pools.

Reference

SW06.W31–9
Polyamine conjugates – a mechanism for selective drug delivery
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Cancer treatment is continually improving but one of the major challenges still remaining is how to deliver the cytotoxic drug selectively to the cancer cell. Selective delivery would minimise the toxicities (side effects) and may improve outcomes. The polyamine pathway has been identified as a useful target for intervention but to date single enzyme inhibitors, polyamine antimetabolites and mimetics have not been successful in the clinic. An alternative strategy is to use the polyamine transport system (PTS) as a means of drug delivery. The PTS is upregulated in cancer cells and because of its broad specificity it can accommodate a variety of toxic agents attached to a polyamine tail. We have investigated a number of polyamine conjugates and assessed their ability to kill different types of cancer cells in comparison with known anticancer drugs and the parent agent. Structure activity relationships have been determined as has potency in different types of cancer. This approach holds much promise with the conjugates showing improved efficacy over the parent drug.

SW06.W31–10
Design of small molecule epigenetic modulators based on the polyamine backbone
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Histone demethylases such as lysine-specific demethylase 1 (LSD1) mediate an important cellular mechanism for epigenetic control of gene expression. In particular, histone H3 dimethyl lysine 4 (H3K4me2) is a transcription activating chromatin mark at gene promoters, and aberrant demethylation of this mark by LSD1 may broadly repress the expression of tumor suppressor genes that are important in human cancer. We, and others, have conducted studies verifying that LSD1 is an exciting new therapeutic target. We reported a series of (bis)guanidines and (bis) biguanides that are potent inhibitors of recombinant human LSD1. These inhibitors significantly increase H3K4me2 levels, initiate chromatin remodeling and induce the re-expression of tumor suppressor genes. The potent LSD1 inhibitor 2d promoted re-expression of multiple, aberrantly silenced genes important in the development of colon cancer, including members of the secreted frizzle-related proteins (SFRPs) and the GATA family of transcription factors. We were the first to demonstrate the antitumor effects of LSD1 inhibitors in vitro and have recently demonstrated their significant antitumor effects in vivo. Based on
this lead, and on a recently conducted virtual screen, we have now identified additional LSD1 inhibitors typified by SKS-89-4C, SKS-89-18 and STH-86-20C. In this presentation, the syntheses leading to these analogues, their characterization as LSD1 inhibitors, their cellular effects and their evaluation as antitumor agents will be discussed.

Hematological characterization of SSAT mice revealed myeloproliferative phenotype with enhanced myelopoiesis and thrombocytopenia as well as anemia. The phenotype progressed with age. The bone microenvironment seemed to have an impact on the development of the myeloproliferative disorder. The maturation and mineralization potential of SSAT osteoblasts was impaired resulting in osteopenic bones. Overall, the hematopoietic and bone phenotype of SSAT mice resembled that of aging humans. CML, AML and ALL patients had higher levels of spermidine and spermine as well as higher SSAT activity in their peripheral blood leukocytes than did their healthy controls. The increased SSAT activity was associated with the severity of the disease in myeloid leukemia types but not in ALL.

In conclusion, we found an association between polyamine metabolism and myeloid leukemia indicating that enhanced SSAT activity predisposes hematopoietic cells to myeloproliferation. The findings support the involvement of aging and bone micro-environment in the proliferation and differentiation potential of hematopoietic cells.

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SW06.W31–12
Potentiation of cytotoxicity of polyamine metabolites induces apoptosis in tumor cells: new approaches in cancer therapy by nanocarriers

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Clonogenic and MTT assays showed that the cytotoxicity induced by bovine serum amine-oxidase (BSAO) and spermine (SPM) was enhanced in LoVo and M14 cell lines, by the pretreatment of tumour cells with lysosomotropic compounds, like chloroquine (CQ) or MDL 72527. These compounds sensitized both wild-type (WT) and multi-drug resistance (MDR) cells to the subsequent exposure to SPM metabolites. The sensitization of the cytotoxic effect was greater in MDR cells than in their WT counterparts. Annexin V assay showed that the combination of BSAO/SPM enzymatic system with CQ or with MDL 72527 had a synergistic effect on cell growth inhibition by inducing apoptosis. A similar enhancement in tumour cells was also observed after the pre-treatment with the aldehyde piperidine oxime-ether hydrochloride (EHW437), a new chemical compound.

Human adenocarcinomas (LoVo) and M14 cell lines were also pre-treated with anandamide (AEA), an endocannabinoid, and then treated with BSAO/SPM. The sensitizing effect was higher on MDR cells than wild-type ones in both cell lines. The results are supported by Annexin V-FITC PI assay. Pre-treatment with AEA increased the percentage of apoptotic cells on both WT and MDR phenotypes. Flow cytometry analyses showed that BSAO and spermine induced a remarkable appearance of subG1 peak, on both cell lines, that was even more increased when cells were pre-treated with AEA.

To increase the stability of the enzyme and the release of cytotoxic products, BSAO was conjugated on a new injectable nanohydrogel (NHs), obtained derivatizing hyaluronic acid (HA) with cholesterol (CH). The HA-based NHs system is a useful controlled delivery system for future therapeutic enzymes application.
Polyamines neurotoxicity at the brain and ways of its correction

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Changes in polyamine levels are associated with aging and CNS diseases. Polyamines are involved in a processes of oxidative stress (OS) induction and development. Result of thse processes is the accumulation in the brain of a product of their oxidation 3-aminopropanal and acrolein, possessing the expressed neurotoxicity.

Natural dipeptide carnosine (-alanyl-L-histidine) is an effective protector against OS.

Both polyamines and their oxidation product acrolein cause the development of the OS in PC-12 cells. For the first time it was established the protective effect of carnosine on cell death and ROS growth which effectiveness was determined by a toxic dose of acrolein and incubation time with its presence, as well as the mode of carnosine administration.

Senescence accelerated mice strain (SAMR1/SAMP1) has shown the polyamine system violation in early ontogeny leading to antioxidant defense system decreased level. In these conditions the reduction of polyamines in the brain tissue is a significant factor for OS development.

SAMP1 mice have high sensitivity to negative effect of acute hypobaric hypoxia, leading to the development of secondary hemic tissue hypoxia. Proton magnetic relaxation study showed that the exposure of SAMP1 mice to hypoxia can cause cerebral edema.

The clinical and biochemical analysis showed a reduction in the PA levels in blood of patients with chronic cerebrovascular diseases. Including carnosine to complex therapy prevents the reduction of PA, which is accompanied by improvement in cognitive function of the brain.

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Polyamines transport by probiotics

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Polyamines, such as putrescine, cadaverine, agmatine, spermine, and spermidine, have been reported in a variety of foods, such as fish, meat, cheese, vegetables, and wines, where they are naturally present [1]. Moreover, they are essential components of all living cells where they exhibit different roles in cellular growth, normal function, proliferation, differentiation of immune cells as well as in regulation of inflammatory reactions. Thus, the regulation of polyamine levels from the diet is important to keep the function of various organs and their body pool is maintained by three forms among women. Thus, the goal of many research studies is being tested both in vitro and in vivo [1].

Cisplatin is one of the most used anticancer drugs in the clinic but its high cytotoxicity and acquired resistance led to the search of new metal-based antitumor agents displaying higher efficacy, reduced toxicity and lack of cross-resistance. Many Pt(II) and Pd(II) coordination compounds have been recognized to display significant anticancer characteristics by covalently bind to DNA bases yielding intra- and interstrand adducts, blocking DNA replication and transcription and finally leading to cell death [2].
In this study, novel Pd(II) and Pt(II) complexes containing analogues of biogenic polyamines as linkers were synthesized. The cytotoxic activity of the ligands and their chelates was evaluated in several human breast cancer cell lines. The potential anti-neoplastic effects and selectivity of these agents on polyamine homeostasis, cancer stem cell population and DNA structure were also examined. The overall data show that treatment with specific compounds results in growth inhibition, cell death induction, changes in polyamine homeostasis, DNA damage induction and phenotypic effects that vary in the different cell lines.

References

**SW06.W31–17**

**Hepatitis C virus alters the polyamine metabolism in human hepatoma Huh7 cells**

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Hepatitis C virus (HCV) is a highly pathogenic virus which induces various types of severe liver damages including steatosis, fibrosis, and hepatocellular carcinoma. HCV replication or expression of its core and NS5A proteins activates production of reactive oxygen species (ROS). It is considered that HCV-induced oxidative stress contributes to the virus-induced liver disorders. Biogenic polyamines spermine and spermidine participate in numerous cellular processes including transcription, translation, RNA processing. In addition, they counteract oxidative stress by direct scavenging of ROS. However, the effect of HCV on polyamine metabolism has never been investigated so far.

Our goal was to investigate if HCV proteins can alter biosynthesis or degradation of biogenic polyamines and to reveal molecular mechanisms underlying such dysregulation.

We showed that transient expression of HCV core and NS5A proteins, which induced oxidative stress, led to up-regulation of transcription of ornithine decarboxylase (ODC), spermidine/spermine-N\(^2\)-acyetyltransferase (SSAT), and spermine oxidase (SMO) genes, the key enzymes of polyamine metabolism. Activation of transcription of SSMAT and ODC genes was accompanied by an increase in the intracellular activity of the corresponding enzymes. Noteworthy, this activation was rather short, and it was followed by 2–5-fold decrease of SSAT and ODC activity, compared to the naïve Huh7 cells. In contrast, in cells, expressing HCV core and NS5A proteins, increase of SMO activity was more prolonged. A long-lasting expression of HCV proteome in a stable cell line harboring the full-length HCV replicon led to a 3–5-fold reduction of ODC and SSMAT activity and to a concomitant decrease in levels of spermine and spermidine.

These data show that HCV can alter metabolism of biogenic polyamines which might account for disturbance of cellular life cycle, suboptimal growth and contribute to proliferation of the infected cells. Further investigation of the relationship between polyamines and HCV can lead to the discovery of new approaches in the therapy of hepatitis C.

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**SW06.W31–18**

**Novel (R)- and (S)-isomers of 3-methylspermidine**


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Biogenic polyamines spermine (Spm), spermidine (Spd) are present in all types of cells in mM–mM concentrations and essential for their normal growth. Investigation of individual cellular functions of Spm and Spd at molecular level is complicated since these polyamines are interconvertable and partly interchangeable.
Metabolically stable and functionally active Spd analogues with such properties are unknown.

Recently, we demonstrated that biological properties of C-methylated Spd’s can be regulated by simple movement of methyl group along Spd backbone. Racemic 3-MeSpd was metabolically stable on the contrary with 1- and 2-MeSpd’s [M.Hyvonen et al., JMC 54, 4611-8 (2011)]. Racemic 3-MeSpd like 1- and 2-MeSpd’s was a substrate of DHS, but on the contrary with the last two didn’t support the growth of DU145 cells with chronic polyamine deficiency [M.Hyvonen et al., Amino Acids 42, 685-95 (2012)].

Basing on the peculiarities of the interaction of (R)- and (S)-1-MeSpd’s with DHS [M.Hyvonen et al. JBC 282, 34700-6 (2007)], one may expect that biologically active isomer of 3-MeSpd doesn’t penetrate inside cells. To check this we obtained early unknown (R) and (S)-3-MeSpd’s.

Commercially available optically active N-Boc-alaninols were used as starting compounds and were transformed to (R)- and (S)-N′-Boc-1,3-diaminobutane. Alkylation of their o-nitrophenyl sulfonyl derivatives with N′-(4-iodobutyl)phthalimide was used to build up the C-N-bond. (R)-isomer of 3-MeSpd is the first metabolically stable functionally active mimetic of Spd, supporting the growth of cells with chronic polyamine deficiency. Interaction of the synthesized isomers with the enzymes of polyamines metabolism and accumulation inside cells will be discussed.

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SW06.W31–19
Aminoxy analogue of histamine is an efficient inhibitor of mammalian L-histidine decarboxylase: combined in silico and experimental evidence
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Histamine plays highlighted roles in the development of many inflammatory, neurological emergent and rare diseases. Intervention of the effects exerted by histamine is currently achieved by using modulators of the activities of the four histamine G-protein-coupled receptors, namely H1 to H4. Histamine is formed decarboxylation of L-histidine, which is catalyzed by pyridoxal-5'-phosphate (PLP) dependent histidine decarboxylase (HDC, EC 4.1.1.22). The reduced availability and stability of the protein have delayed the characterization of its structure-function relationships. It has been a handicap to design intervention strategies based on histamine synthesis inhibition. HDC could be an interesting target for intervention of multiple human diseases. Our previous knowledge (derived from both in silico and experimental approaches on rat HDC) encouraged us to disclose some structure-activity relationships being of importance for design of novel specific inhibitors of the enzyme. Our in silico (virtual screening) results indicated that an effective competitive inhibitor should be capable to form an ‘external aldimine-like structure’ and to have an imidazole group, or its proper mimetic, to provide additional affinity of PLP-inhibitor adduct to HDC active center. This work is supported by Presidium RAS project #12-HL-4-1047 and RFBR grant #11-04-99001.

SW06.W31–20
Regulatory effects of polyamines on E. coli persister cell formation and heterogeneity of persister subpopulation
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Background: On the basis of our previous data demonstrating upregulation of E. coli survival to antibiotics by polyamines we assumed that this effect could be at least partially conditioned by implication of polyamines in persister cell formation.

Objectives: 1. Studying the dependency of persister cell formation on polyamines. 2. Testing the structure of persister subpopulation for tolerance to netilmicin.

Methods: High concentrations of netilmicin (several tens of MIC) were used to monitor dynamics of persister cell number in E. coli batch cultures. HPLC fluorescent method was used for determination of cell polyamines. rpoS::lacZ operon fusion was transferred to E. coli HT306 with λRZ5. Gene expression was determined by β-galactosidase activity. Western blot was used to assess the quantity of cell RpoS protein.

Conclusions: E. coli batch cultures of polyamine-requiring mutant demonstrated polyamine-dependent increase in persister cell frequency by three orders of magnitude during the cell transition to stationary phase. This concentration-dependent polyamine effect was mediated by the upregulation of cell RpoS level. When polyamine-supplemented batch cultures were tested for persister cell number with several netilmicin concentrations, the heterogeneity of subpopulation of persister cells was revealed. The wave-like dynamics of persister frequency were significantly dependent on the level of tolerance specific for the particular persister fraction. The first wave of the least tolerant fraction of persister cells was characterized by the highest frequency and the shortest time required to reach its maximum. However, the increase in tolerance of persister cells exhibited elongation of the time required to reach the maximum persister frequency during the stationary phase and lowering their peak values.

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SW06.W31–21
Selective acetylation of primary amino groups: simple method to prepare N,N'-diacetylated polyamines for biological studies
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Naturally occurring polyamines are required for mammalian cell growth. Their concentrations can be at millimolar levels in rapidly proliferating cells and the changes in the homeostasis of the natural polyamines is linked to various pathological condi-
tions, such as acute pancreatitis, diabetes and cancer. One of the main catabolic reactions of natural polyamines is N-acetylation by Spermidine/Spermine N\textsuperscript{\textbeta}-Acetyltransferase and the mono- or di-acetylated polyamines can be further exported from cells and excreted from body. Impairment of the physiological regulatory mechanisms of cell proliferation ultimately leading to cancer has been shown to increase the amount of polyamines that are excreted to urine and further studies have shown that N\textsuperscript{\textbeta},N\textsuperscript{\text\textalpha}-diacetyl-Spm (DASpm) might be a suitable biomarker of cancer.

Currently, the synthetic methods to prepare acetylated polyamines rely on the use of orthogonal protection and step-by-step elongation of the polyamine backbone. While using this traditional way one can reach high purity compounds, the synthesis is time consuming and requires a lot of reagents. We have recently developed selective synthetic methods for acetylation of primary amino groups that do not require any catalyst. These kinds of acyl transfer reactions are already known but have before required the use of catalysts such as pincer-ruthenium complex [1] or carbene [2]. The other option has been to use catalysts with additives (such as 1,8-diazabicyclo[5.4.0]undec-7-ene) [3] that can be expensive and difficult to remove from the reaction mixture. Our reaction shows high selectivity for primary amino groups over primary hydroxyl— or secondary amino groups and can be performed in room temperature with high yields. Furthermore, it is very atom efficient way to synthesize diacetylated polyamines with only one step, and thus, simplifies complex synthesis of these compounds.

References

SW06.W31–22
Exogenous polyamines as inducers of beta-lactam biosynthesis in A. chrysogenum
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Novel data indicates, that biogenic polyamines – 1,3-diaminopropan-1e (1,3-DAP) and spermidine (SP) can increase the expression of beta-lactam biosynthetic genes in fungi [Martin J., et. al., 2012]. These effects are mediated by LaeA – a global transcriptional regulator of filamentous fungi, controlling epigenetically expression of fungal secondary metabolism and differentiation genes by heterochromatin reorganization [Brakhage A., et. al., 2013].

We performed comparative analysis of the influence of exogenous polyamines on the dynamics of cephalosporin C (CPC) production and gene expression in industrial and laboratory strains of A. chrysogenum ATCC11550 and A. chrysogenum VCM F-4081, differing more than hundred-fold in the rate of CPC biosynthesis. CPC biosynthesis was stimulated by 1,3-DAP and SP in both strains. This increase correlated with up-regulation of known ‘early’ CPC biosynthesis genes pchAB, pchC, cepD1 and cepD2, studied at several time-points in the course of cultivation.

To get insight the mechanisms of this stimulation we also analyzed A. chrysogenum orthologs of LaeA and SAMeDC (encodes S-adenosyl-L-Methionine decarboxylase – key enzyme, responsible for SAMe homeostasis). LaeA is a S-adenosyl-L-Methionine (SAMe)-dependent histone methylase, its activity should correlate with intracellular levels of SAMe – the universal donor required for methylation of nucleic acids, phospholipids, and proteins. SAMe levels in turn are tightly linked both to polyamine homeostasis, since SAMe is the only one precursor for spermine and spermidine biosynthesis, and to methionine metabolism.

This possible balance between polyamine, methionine levels and epigenetic control of secondary metabolism may explain the so-called ‘methionine puzzle’ – known for decades but still unclear positive regulation of exogenous methionine on the production of CPC in A. chrysogenum.

Data of the effects for exogenous SP and 1,3-DAP on CPC biosynthesis and gene expression in two A. chrysogenum strains will be presented.

SW06.W31–23
Synthesis of fluorescent derivatives of short-chain polyamines for monitoring of intracellular processes
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Such polyamines as spermine and spermidine are widely known as the important substances in life cycle of many organisms including mammals and microbes. Methylated polyamines have been founded in siliceous frustules of diatom algae and in siliceous sponges. Physiological role of the biogenic polyamines is not clear enough, especially in the case of silicifying organisms. New fluorescent-tagged polyamines are urgent as agents for tracking the natural polyamines and also for visualization of amine-conjugated processes in living cells.

Our synthetic procedures are based on the previously developed step-wise approaches to methylated oligopropylamines bearing one or two terminal NH-groups. We have obtained a collection of dyes with 7-nitro-2,1,3-benzoxadiazole, fluoresceine and rhodamine moieties. Polyamine chains contain 1-4 nitrogens. Fluorescent-tagged polyamines having one terminal NH-group are useful agents for synthesis of more complicated structures, e.g. fluorescent polymers.

The following results were obtained using new fluorescent derivatives of polyamines:
• First stage of silicic acid assimilation by diatom Synedra acus was studied and formation of submicrometer silicon-containing particles in the cytoplasm was found for the first time. These particles exist during the initial stage of siliceous valve growth and they are not observed during further valve maturing;
• S. acus growth in the presence of fluorescent-tagged poly (acrylic acid) resulted in fluorescent siliceous valves. This is the first experimental evidence of hypothesized pinocytosis mechanism supposed for silicon assimilation from the environment;
• Cultivation of siliceous sponges with the addition of fluorescein-tagged polyamines is accompanied by staining of growing siliceous spicules. This finding opens new ways for study of bi silica formation by sponges;
• Fluorescent diatom valves and sponge spicules are promising fluorescent materials obtained by biotechnology.
• The study was partially supported by Project # 11-04-00707-a of the Russian Foundation for Basic Research.
**SW06.W31–24**

Quantitative analysis of natural polyamines from human urine by using LC-MS/MS

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Metabolic studies play an important role in novel drug discovery and clinical diagnostics. Several methods have been developed, e.g. based on quantitative NMR spectroscopy [1] that allows quantification of hundreds of compounds, like lipids and amino acids, directly from serum or urine without any additional derivatization. The natural polyamines (PAs) are essential for mammalian cell growth and differentiation, but the amounts of these compounds in body fluids are low and thus their quantification is typically based on indirect methods requiring pre- or post-column derivatization. The main drawbacks associated with derivatization are the elongated analysis times, low reproducibility, interferences problems and instability of derivatives [2].

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) represents a powerful tool for metabolite profiling studies since it can perform metabolite identification, as well as structural characterization and quantitation of the metabolites [3]. However, to achieve acceptable reproducibility and reliability, quantitation by MS/MS methods require labeled internal standard for each analyte to correct for the variations in sample preparation and to compensate for the variability in MS detection.

In this study we developed atom effective synthetic methods to prepare the most common natural PAs (Dap, Put, DiAcPut, Cad, AcCad, DiAcCad, Spd, N¹-AcSpd, N⁶-AcSpd, DiAcSpd, Spm, AcSpm, DiAcSpm) without and with deuterium labeling. Reference compounds were used for quantification of PA levels from human urine samples taken from patients with prostatic cancer, hyperplasia and other urological cancers (bladder, kidney) by the developed LC-MS/MS method. Preliminary results will be shown during the poster session.

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**References**


**SW06.W31–25**

Loss of mutant K-RAS leads to suppression of invasion and metastases in pancreatic cancer cell lines

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies characterized by the local invasion into surrounding tissue and early metastases into distant organs. Oncogenic mutations of the K-RAS gene occur in more than 90% of all human pancreatic cancers. The goal of this study was to investigate the functional significance and downstream effectors of the mutant K-RAS signaling in the pancreatic cancer invasion and metastasis. We applied the homologous recombination technique to stably disrupt the mutant K-RAS oncogene in the human pancreatic cell line MiaPaCa-2 which expresses an activated K-RAS G12C oncogene. We found that clones with inactivated mutant K-RAS gene exhibited low RAS activity, reduced growth rates and reduced motility and invasiveness in in vitro assays. In vivo, MiaPaCa clones with disrupted mutant K-RAS displayed the reduced tumor formation ability and increased survival rates. We attributed the suppression of tumor invasion and metastasis in mutant K-RAS inactivated clones to the alterations in K-RAS signaling cascade involving Rho-GTPase-activating protein 5 and cavelolin-1. Since polyamines are important for pancreatic tissue maintenance and repair and involved in the cell migration and invasion, we specifically assessed the consequences of mutant K-RAS inactivation for polyamine metabolism. The K-RAS inactivation resulted in the downregulation of a rate-limiting enzyme in the biosynthesis of polyamines ornithine decarboxylase (ODC) through the suppression of the ODC transcriptional regulator c-MYC oncogene. Furthermore, induction of the spermidine/spermine acetyltransferase 1 (SSAT) gene was observed upon loss of the K-RAS activity in MiaPaca-2 clones. In the SCID mouse orthotopic pancreatic cancer model, treatment with an irreversible inhibitor of ODC, a-difluoromethylornithine (DFMO, 500 mg/kg/day by oral gavage) did not have an effect on the survival rates of the mice injected with the MiaPaca-2 cells, but significantly improved the survival rates of mice injected with the mutant K-RAS inactivated MiaPaCa clones. These data indicate that the activated K-RAS gene plays a key role in pancreatic tumorigenesis by altering cell turnover and migration through regulation of genes involved in growth, migration and invasion. This study also provides support for a therapy involving DFMO in combination with other downstream targets of mutant K-RAS oncogene for the management of the pancreatic cancer disease.

**SW06.W31–26**

Insights into the interaction of an agmatinase-like protein with Mn²⁺ and Zn²⁺ ions


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Agmatine, a precursor for polyamine biosynthesis, is also associated with neurotransmitter, anticonvulsant, antineurotoxic and antidepressant actions in the brain. It results from decarboxylation of L-arginine by arginine decarboxylase and it is hydrolysed to urea and putrescine by agmatinase. Recently, we have described a new protein, immunohistochemically detected in the hypothalamic region and hippocampal astrocytes and neurons of the rat brain, which also hydrolyze agmatine although its sequence greatly differ from all known agmatinas. This agmatinase-like protein (ALP) exhibits a LIM-like domain close to its carbboxyl terminus, whose removal results in a truncated variant with a 10-fold increased kcat and a 3-fold decreased Km value for agmatine. The isolated, purified LIM-domain, which contains 2 Zn²⁺/domain, resulted to be inhibitory to truncated species, but not to the wild-type enzyme. Our proposal was that the LIM-domain functions as an autoinhibitory, regulatory entity for ALP. We have now examined the interaction of ALP with the catalytically required Mn²⁺ and the LIM-associated Zn²⁺, by using mutagenic and kinetic approaches. The C492A variant was shown to be a Zn²⁺-free enzyme with kinetic parameters similar to those of the truncated-ALP (increased catalytic activity). With regard
Polyamine oxidases (PAO), a FAD-containing enzymes are involved in the biodegradation of polyamines, catalyzing their oxidative deamination. Polyamine oxidation products—aminoaldehyde—are cytotoxic and have been considered as a cause of apoptotic cell death due to their ability to act as the carbonyl agents, conjugate with proteins and nucleic acids. Histochemical studies of animal and human malignant tissues showed the decrease of aminoxydaze activity in N-nitrosoaldamines-induced hepatomas, Guerin’s carcinoma, human nephroblastosomas and lymphomas and other cancer types. The catalysis of polyamines by the oxidative deamination is decreased or almost arrested in tumor cells. Thus, the activators of polyamine catabolism are supposed to be potential antineoplastic agents.

The influence of aniline, azo fluorine, benzimidazole, and dioxidoreineoimidopyridine derivatives on the rate of putrescine, spermidine and spermine oxidative deamination were evaluated in the acellular system of the rat regenerating liver. Only azoflurone and aniline compounds were activators of polyamine catabolism, especially: 1-amino-9-phenylamino-4-azofluorene, 1-amino-2-bromo-4-azofluorene-9, 3(4-iodinanilino)-1-phenylprop-29e-1, 3(1-phenyl-2-fluorinanilino)-propionate-1. The antiproliferative activity was examined in prostate cancer cell line PC-3 by vitality test with Alamar Blue. QSAR analysis with Chemi-Descrip software demonstrated the significant correlation of cytotoxicity with the Balaban topological index ($R^2 = 0.7$) but not with Detour index. Docking with yeast PAO enzyme Fmsl using Molegro Virtual Docker software was performed with flexibility in torsion angles of ligands. The lowest energy pose of each compound was described by energies of ligand interaction with different residues of enzyme. Decomposition of total docking score into partial ligand-residue terms was performed using the ‘energy inspector’ tool embedded in MVD. There were identified few critical atoms in the sphere radius 12A of the PAO active center: His67, Tyr540, His191, Trp174, Gll487.

Results revealed the structural basis for the design and synthesis of novel activators of polyamine catabolism as potential antitumor agents.

**SW06.W31–28**

**Structural aspects of Polyamine oxidase activation by azo fluorene and aniline derivatives**

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Polyamine oxidases (PAO), a FAD-containing enzymes are involved in the biodegradation of polyamines, catalyzing their oxidative deamination. Polyamine oxidation products—aminoaldehydes—are cytotoxic and have been considered as a cause of apoptotic cell death due to their ability to act as the carbonyl agents, conjugate with proteins and nucleic acids. Histochemical studies of animal and human malignant tissues showed the decrease of aminoxydaze activity in N-nitrosoaldamines-induced hepatomas, Guerin’s carcinoma, human nephroblastosomas and lymphomas and other cancer types. The catalysis of polyamines by the oxidative deamination is decreased or almost arrested in tumor cells. Thus, the activators of polyamine catabolism are supposed to be potential antineoplastic agents.

The influence of aniline, azo fluorine, benzimidazole, and dioxidoreineoimidopyridine derivatives on the rate of putrescine, spermidine and spermine oxidative deamination were evaluated in the acellular system of the rat regenerating liver. Only azoflurone and aniline compounds were activators of polyamine catabolism, especially: 1-amino-9-phenylamino-4-azofluorene, 1-amino-2-bromo-4-azofluorene-9, 3(4-iodinanilino)-1-phenylpropionate-1, 3(1-phenyl-2-fluorinanilino)-propionate-1. The antiproliferative activity was examined in prostate cancer cell line PC-3 by vitality test with Alamar Blue. QSAR analysis with Chemi-Descrip software demonstrated the significant correlation of cytotoxicity with the Balaban topological index ($R^2 = 0.7$) but not with Detour index. Docking with yeast PAO enzyme Fmsl using Molegro Virtual Docker software was performed with flexibility in torsion angles of ligands. The lowest energy pose of each compound was described by energies of ligand interaction with different residues of enzyme. Decomposition of total docking score into partial ligand-residue terms was performed using the ‘energy inspector’ tool embedded in MVD. There were identified few critical atoms in the sphere radius 12A of the PAO active center: His67, Tyr540, His191, Trp174, Gll487.

Results revealed the structural basis for the design and synthesis of novel activators of polyamine catabolism as potential antitumor agents.

**SW06.W31–29**

**Isosteric analogues of natural polyamines with altered carbon chain length and additional amino groups**

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The cationic biogenic polyamines spermine (Spm) and spermidine (Spd) interact with negatively charged components of the cells and are essential for their growth and viability. Spm and Spd are positively charged at physiological pH (7.4) and the localization of the charges defines their recognition. Alteration of the carbon chain length and addition of extra amino group(s) into polyamine backbone gives rise to isosteric analogues with reduced
degree of protonation. The total net charge of 1,12-diamino-3,6,9-triazadodecane (SpmTrien), which is Spm isoster, is +3 at physiological pH [1], while 1,8-diamino-3,6-diazaoctane (Trien), which is Spd analogue, is +2. Despite these analogues have different localization and total net charge they still have some of the properties of their parent polyanines.

Trien, being an excellent chelator of Cu²⁺ ions, is used to treat Wilson's disease when person is intolerant to penicillamine and recently Trien was shown to ameliorate left-ventricular hypertrophy in type 2 diabetic patients [2]. Trien is a poor mimetic of Spd: it penetrates poorly inside cells, and cannot fulfill most of Spd cellular functions. However, Trien exhibits some substrate properties of spermidine/spermine N'-acetyltransferase (SSAT1) reaction [3], but its physiological acetylating enzyme is thialysine N-acetyltransferase [4]. SpmTrien is more successful mimetic of Spm: it efficiently penetrates inside cells and can fulfill some of Spm cellular functions [3]. In cells and in vivo SpmTrien is acetylated by SSAT1 and then oxidized by acetylpolyamine oxidase to Trien [3,5]. Therefore SpmTrien can be considered as a bioactive precursor of Trien.

References

**SW06.W32 Biochemistry of Invertebrates (VI-W32)**

**SW06.W32–1**

**Blue blood: structure, evolution and function of hemocyanins**

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Hemocyanins are the blue respiratory proteins in many mollusks and arthropods. They have copper in their active site and are large extracellular protein complexes. Hemocyanins bind oxygen cooperatively, with Hill coefficients up to 10 in some arthropods. Molluscan and arthropod hemocyanins evolved independently from tyrosinase-like ancestral oxygen-binding proteins. In recent years the evolution and quaternary structure of different hemocyanin types have been elucidated by a combination of DNA sequencing, X-ray crystallography and 3D electron microscopy. According to molecular clock calculations, arthropod hemocyanin evolved <550 million years ago, whereas molluscan hemocyanin originated ca. 740 million years ago.

Arthropod hemocyanins are cubic hexamers (1x6mers) or oligo-hexamers (2x6mers, 4x6mers, 6x6mers, 8x6mers) of 75 kDa polypeptide subunits. The 8x6mer has approximately the size of a ribosome (25 nm). Different subunit types play distinct roles in the oligo-hexameric assemblies.

Molluscan hemocyanins are semi-hollow cylinders 35 nm in diameter, consisting of an outer wall and an internal collar. This quaternary structure is based on different 50 kDa functional units that are arranged, within the polypeptide subunit, like a pearl chain. The first six of these functional units (termed FU-a through FU-f) make up the cylinder wall which is rather invariant. In contrast, the internal collar has experienced considerable variation in that additional collar functional units evolved or disappeared.

High resolution 3D reconstructions of the quaternary structure of various hemocyanins are now available and will be show.


**SW06.W32–2**

**Metabolic regulation and energy balance in stress tolerance of marine bivalves: Integrating the effects of multiple stressors**

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Understanding the effects of multiple stressors in environmentally realistic context is a grand challenges of marine biology. Bioenergetic approach provides a useful tool to integrate the effects of multiple stressors and predict their ecological consequences. Environmental stress can affect the energy balance of an organism resulting in trade-offs between basal maintenance of a stressed organism and energy costs of fitness-related functions such as reproduction, development and growth. Our studies in marine bivalves (eastern oysters *Crassostrea virginica*, hard clams *Mercenaria mercenaria* and blue mussels *Mytilus edulis*) showed that a bioenergetic plays a key role in setting limits of tolerance to a variety of environmental stressors including temperature, salinity, elevated CO₂ levels and metal pollutants. Exposure to these stressors and their combinations resulted in elevated energy demand reflecting upregulation of cellular protection mechanisms such as metal-binding proteins, molecular chaperones and antioxidants. Combined exposure to elevated CO₂ and metals also affected protein degradation systems potentially altering protein stability and turnover. Increasing energy demand for stress protection and damage repair in bivalves exposed to combinations of elevated temperature, CO₂, reduced salinity and trace metals led to a progressive mismatch between energy demand and aerobic energy supply of the bivalves, and eventually to energy deficiency, reduced growth and survival. Trace metals such as Cd and Cu exacerbated this energy mismatch by inhibiting aerobic and/or anaerobic ATP producing pathways. Oxidative stress was observed in response to extreme stress or during early phases of response to moderate stressors but was typically compensated during chronic sublethal stress, in line with the highly evolved stress protection mechanisms in these intertidal species.

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**SW06.W32–3**

**Modular organization of arachnid toxins**

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Arachnids, and more specifically spiders and scorpions, produce potent venoms serving to subdue prey or fight off aggressors. Arachnid venoms are sources of invaluable chemicals that are nowadays routinely used as research tools in neurobiology. Most studied arachnid venoms are enriched in peptides (usually 30–70 residues) that come in two flavors: neurotoxins and cytotoxins. Neurotoxic peptides typically contain 3–4 disulfide bridges, the ICK fold being characteristic of spider toxins and the CSβ fold commonly found in scorpions. To the contrary, cytotoxins are usually linear cationic membrane-active peptides. Both types are quite simple single-domain molecules.

Extensive studies revealed that arachnids evolved to produce more complex toxins with modular organization. In spider ven-
oms, for instance, numerous two-domain polypeptides were discovered, each domain corresponding to a simple single-domain toxin. It is of note that all four possible combinations of domains were found: ICK-ICK, ICK-linear, linear-ICK, and linear-linear. Such elaboration resulted in emergence of novel modes of action, which in turn were selected in evolution. For example, the linear-linear two-domain toxins acquired unprecedented insect toxicity. The ICK-linear and linear-ICK combinations present the ‘membrane-access’ mechanism of action, and the domains in ICK-ICK toxins feature positive cooperativity.

A different type of modular design is noted in some scorpion toxins. The so-called α-toxins that slow down inactivation of voltage-gated sodium channels are single-domain Cαββ peptides. It was shown, however, that they are composed of two subdomains: the more conserved and rigid ‘core module’ and flexible and variable ‘specificity module’, which determines toxin specificity. In this case modular structure in toxins evolved to match the domain architecture of target channels.

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SW06.W32–4
Glutathione S-transferase activity and glutathione level in the cestode Eubothrium crassum, Salmonid’s parasite
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It is known that cestodes live in hosts intestines which present an aggressive environment. It means that helminthes must have adequate system of biochemical defense. The glutathione S-transferase (GST) superfAMILY enzymes and reduced glutathione (GSH) are essential part of organism defense system against numerous endogenous and exogenous toxins and products of oxidative stress.

In this study GST activity with universal substrate 1-chloro-2,4-dinitrobenzene (CDNB) was estimated to be about 24 nmol/min/mg protein, which is significantly lower (2.8–28-fold) in comparison with GST activity in host tissues (trout Salmo trutta). GST activity with substrate 4-nitroquinoline N-oxide (NNO) was about 8 nmol/min/mg protein, that is similar to data received for trout tissues. Concentration of GSH in helminth’s tissue was about 1.51 μg/mg protein, 1.6–38-fold higher than in host tissues. We suppose that obtained differences between helminthes and their hosts are related with organism’s environment. Helminthes constantly live in micro surrounding of manifold metabolic products stimulated oxidative stress. Pathogenic effect can be eliminated by synthesis of the high level of GSH and GST activation, because it’s key role in defense against oxidative stress.

SW06.W32–5
Changes of blue mussels Mytilus edulis L. non-methylene-interrupted fatty acids content in response to environmental effects
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Marine invertebrates such as bivalve mollusks contain non-methylene-interrupted fatty acids (NMI FA) particularly 20:2 Δ5,11 and 20:2 Δ5,13 and their chain elongation products 22:2 Δ7,13 and 22:2 Δ7,15. Bivalves are able to synthesize de novo the NMI FA whereas it is also established that they have limited or no ability to synthesize polyenoic fatty acids n-3 and n-6 series. Different investigated species of White Sea (Kandalaksha Bay) bivalve mollusks (Mytilus edulis, Modiolus modiolus and Hiatella arctica) contain NMI FA. The high level of the fatty acids (up to 13.7% of sum FA) are in the mussel tissues exposed most often to the environmental effects (gills, distal part of mantle and food) compare with the main metabolic organ such as digestive gland.

The physiological functions of the fatty acids still remain understudied. NMI FA of the membrane lipids make them more resistant to oxidative processes and microbial lipases than usual PUFAs. We investigated effect of some environmental factors on the fatty acid composition (temperature, salinity, anoxia, heavy metals, allelopathic interactions) to determine the possible role of the fatty acids with isolated double bounds in mussels acclimation and their stress responses to different effects. In aquarium experiments the effect of salinity (25 ppt as a control, desalination to 5 ppt and high salinity up to 45 ppt), temperature (15°C as a control, low temperature 5°C and high temperature 20°C) and the toxic products of Halichondria panicea excretion on the fatty acid composition in Mytilus edulis L. were studied (the exposition was 14 days). Acute stress response of mussel lipid content was investigated at the tests of short-term anoxia (12 and 24 h) and heavy metals (cadmium and copper) accumulation (24 h exposition). Increased level of NMI FA indicates that desalination to 5 ppt as well as low temperature (5°C) activates biosynthesis of the fatty acids with isolated double bonds. On the contrary, we observed decreased concentrations of NMI FA under high salinity (45 ppt) and high temperature (20°C).

The influence of the toxic products of Halichondria panicea excretion on the Mytilus edulis fatty acid composition has resulted in increase the NMI FA level after 24 h exposition whereas acclimation period (14 days exposition) had no effect on them. Acute fatty acid composition response on the short-term anoxia (12 and 24 h) and cadmium and copper accumulation (24 h) includes the activation of NMI FA synthesis in mussels. Thus, the elevated content of NMI FA suggests the activation of the synthesis of these acids with unusual structure in the stress ambient conditions. Due to their typical properties the NMI FA have a lower autooxidation rate and prevent oxidative damage to membranes, maintaining membrane fluidity of mussel gills under different environmental effects. Moreover in some cases we observed correlates of n-3 and n-6 PUFAs deficiencies and high amount of NMI FA in stress responses of mussel gill fatty acid compositions. It’s assumed the ability to synthesize NMI FA is one of the adaptive mechanisms of the membrane fluidity maintenance in Bivalves which is incapable of usual PUFA biosynthesis.

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Abstracts

**SW06.W32–6** Understanding the proteolysis of a lifespan regulator protein vitellogenin in honeybee workers

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Vitellogenin is a multitask protein mostly associated with its egg-yolk function. It transports lipids to the egg, where it is proteolytically processed and provides a source of amino acids for the embryo. However, it is also expressed in non-reproductive individuals (workers) of social insects. It is either secreted into hemolymph or stored in fatbody. In the honey bee workers, vitellogenin is a hormone-like protein. It is a central life-history regulator that supports immune cell viability, protects against oxidative stress, and suppresses risky foraging behavior. Our data suggests that vitellogenin is also involved in inflammation. The vitellogenin-induced signaling pathways, however, are little understood so far. Our previous work shows that in the fatbody, vitellogenin is specifically cut into two pieces. This proteolysis could be an important step in vitellogenin signaling. First we characterized the cutting site using homology modeling, NMR and mass-spectrometry. Next, we assayed protease inhibitors in search for the cutting enzyme, and did immunofluorescence microscopy to track the subunits of the protein in honeybee tissues and in an insect cell line. As a result, a phosphorylation-regulated domain linker was identified as the cutting site. We found evidence for a putative vitellogenin-related inflammation pathway. We propose that this pathway is connected to how vitellogenin can affect multiple traits from behavior to immunity in the honeybee.

**SW06.W32–7** Effect of heavy metals and temperature on the activity of some lysosomal enzymes of the White Sea mussels M. edulis

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In recent years the local pollution of individual sections and littoral zones of the White Sea by toxicants, including heavy metals has resulted in changes of the White Sea ecosystem. Physiological and biochemical mechanisms of adaptive responses to heavy metals as well as temperature changes still remain understudied.

We have chosen mussels as model-organisms as they represent the typical mariculture of the White Sea and can be used to release biochemically active compounds for the purpose of applying them in different economic sectors: agriculture, cosmetic and food industry, medicine, bioengineering etc. The mussels are also used as tests to test natural water quality and adaptive responses of aquatic organisms.

In the present study effect of temperature and different concentrations of Cu and Cd salts (chlorides) on the activity of two lysosomal glycosidases (β-glucosidase, β-galactosidase), two lysosomal nucleases (DNAse, RNAse) and acid phosphatase in digestive gland and gills of the White Sea mussels M. Edulis were investigated.

It was shown that the content of Cd in mussels has increased 25–29 times and the content of Cu – 3–4 times during 3-day exposition. The accumulation of heavy metals caused increase of total protein. The high concentration of Cd and Cu caused decrease the activity of enzymes studied in digestive gland while increase their activity in gills.

It was investigated that the activity of lysosomal glycosidases decreased in digestive gland and increased in gills within the range of 0 – +8°C during 1-day exposition. The activity of two lysosomal nucleases and acid phosphatase increased within the range of 0 – +8°C both in gills and digestive gland during 24 h. Temperature decrease within the range of +8–+4°C led to increase of the activity of β-glucosidase, β-galactosidase, acid phosphatase and decrease of the activity of RNAse, DNAse and the content of protein during 1-day exposition.

This study demonstrated different mechanisms of biochemical adaptation on enzyme level to anthropogenic and abiotic factors.

**SW06.W32–8** Production in insect cell lines and functional characterization of mite and whitefly esterases implicated in insecticide resistance

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Esterases are often implicated in insecticide resistance in agricultural pests and insects of public health. The most common mechanism may involve enhanced sequestration and detoxification of the insecticide, via overexpression of esterases that can bind and hydrolyse insecticides, and it has been well studied in organophosphate resistant Culex mosquitoes and aphids. Esterases have been also associated with insecticide resistance against additional insecticides, such as ketoens and pyrethroids in spidermites (Tetranychus urticae) and whiteflies (Bemisia tabaci), by transcriptomic studies.

We have used a plasmid-based expression system to drive expression of whitfly and mite esterases that are implicated in insecticide resistance in insect cell lines. Mite esterases with engineered C-terminal MycHis-tag were efficiently secreted to the extracellular medium from which they could be purified by affinity chromatography. By contrast, whitely BtCoE1 accumulated as a soluble protein in the cytoplasm of the transfected cells. Purified proteins and extracts from transformed cell lines are currently being used in enzyme assays to determine hydrolyase activities against implicated insecticides. In addition, plant extracts will be screened for the presence of compounds that inhibit esterase activities.

**SW06.W32–9** Transcriptome and small RNA analysis of larval midgut tissue persistently and acutely infected by cytoplasmic polyhedrosis virus (CPV) in the silkmoth Bombyx mori

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Insect cytoplasmic polyhedrosis viruses (CPVs) are segmented dsRNA genome viruses that belong to the genus Cypovirus of the family Reoviridae. While the involvement of the RNAi machinery to control infections of ssRNA genome viruses is well established, nothing is known regarding the interaction between cypovirus infection and the RNAi machinery in insects.

In the silkmoth Bombyx mori, BmCPV is a major pathogen which can cause serious damage to the sericulture industry. Interestingly, silkmoth strains such as Daizo and P50 were observed to be persistently infected by BmCPV. Viral genomes could not only
be detected in the midgut epithelium, but also in epidermis, gonads and eggs. It is hypothesized that the persistent BmCPV infection in silkworm tissues results in an altered innate immune response and alterations in the functioning of the RNAi machinery.

In this study, next-generation sequencing techniques were used to determine the transcriptome and small RNA profile of persistently and acutely infected midgut epithelia of silkworm Daizo larvae. These sequencing data aim to elucidate the innate immune response against BmCPV infection and its recognition by the RNAi defense machinery.

As an ultimate goal, these studies attempt to clarify the natural function of the RNAi pathway in the silkworm, thus aiming for the development of more effective approaches to RNAi-mediated inhibition of gene expression in lepidopteran insects.

SW06.W32–10
Characterization of a lef8 knock-out BmNPV: new data for an old gene
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BmNPV is an insect infecting closed-circular dsDNA virus with a host spectrum restricted to the silkworm, Bombyx mori. As a model member of the Nucleopolyhedrosis Virus (NPV) family, it exhibits the typical 3-phase gene expression profile with early, late and very late transcripts and a virus-encoded multi-subunit RNA polymerase, required for the transition from the early to later gene expression. The lef8 gene, which encodes a subunit of this polymerase, is the target of this study: using the virus in baculovirus (AcNPV) mid form, we replaced the lef8 ORF with a zoecon resistance-YFP-reporter element by homologous recombination, thus creating a Δlef8 knock-out BmNPV. Previous data from a temperature-sensitive mutant of lef8 had suggested that the pathogenic capacity of the virus is disrupted at the restrictive temperature, while its replication competence is retained at levels similar to wild-type (wt). We now show that when the entire ORF is removed from the viral genome, the viral replication capacity is also abolished, leaving the Δlef8 bacmid as a persisting entity in Bombyx Bm5 cell cultures up to the 160 h post-transfection. Rescue of the wt phenotype by administration of LEF8 through extra-viral LEF8 gene-constructs (episomal or cell genome-integrated) or ectopic production from within the viral genome was not possible. Only restoration of the ORF (native or N-Mye tagged either under heterologous promoter control or under the control of its native promoter sequences) in its original locus was able to effectively rescue the virus. These findings suggest a previously unidentified position-related function of the lef8 ORF.

SW06.W32–11
Organ-specific distribution of copper, tyrosinase, and SOD1 in fresh-water mollusk Planorbarius corneus
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There are at least two cuproenzymes that participate in the innate immune response in mollusks: tyrosinase, which induces formation of ROS, defending from parasites, and SOD1, protecting host cells from the action of ROS. However the copper metabolism of mollusks is studied insufficiently. In the presented work, copper, tyrosinase and SOD1 distribution in tissues and organs (haemolymph, hepatopancreas, gonad, foot, and head) of fresh-water mollusk Planorbarius corneus was investigated.

According to FAAS the atomic concentration of copper decreases in the order: gonad>hepatopancreas>head>foot>haemolymph. According to biochemical and histochemical analyses distributions of tyrosinase and SOD1 correspond to the profile of copper distribution in the mollusk body. It was shown that even low concentration of copper ions is toxic for mollusks, and the atomic content copper in bodies didn’t change. We also used ions of Ag(I) for studying the copper turnover in mollusk, because Ag (I), being isoelectronic to Cu(I), can be coordinated by copper-binding motives of copper transporting proteins and transferred through copper metabolic system. It was shown that tissues accumulate (Ag(I) in an exchangeable form. The approaches to study copper metabolism in mollusks, its phylogenetic features and as well as its role in realization of protective reactions are discussed.

SW06.W32–12
Effect of recombinant Drosophila Yorkie and Scalloped proteins on intestinal stem cell proliferation
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Intestinal stem cells (ISCs) in adult Drosophila midgut are essential for maintaining gut homeostasis and replenishing cells that are non-functional due to gut damage. In this study, we investigated the signaling pathway that regulates the proliferation and differentiation of ISCs. Previous studies have shown that the Hippo (Hpo) signaling pathway includes the Hippo, Yorkie (Yki), and Scalloped (SD) genes that jointly regulate cell growth, proliferation, and apoptosis. We showed that the Hpo signaling pathway was involved in the proliferation and differentiation of ISCs by Yki and SD RNA interference (RNAi) lines. The number of ISCs in the RNAi lines was lower than that in wild-type flies, after the flies were fed dextran sodium sulfate (DSS), a gut damaging reagent. To analyze the functions of Yki and SD in damaged gut cells, we expressed, purified, and characterized Yki and SD proteins in S99 cell lines. We constructed baculovirus transfer vectors for Yki and SD (pBac1-Yki and pBac1-SD, respectively). Then, the S99 cells were infected using each recombinant baculovirus (vAc-Yki and vAc-SD). Five days after infection, the cells were harvested and analyzed using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) or western blotting. The functions of the recombinant proteins Yki and SD were determined using MTS cell proliferation assay and evaluating the survival rate. Not only did the Drosophila melanogaster insect cell line (D-mel2) and primary Drosophila intestinal cell numbers increase, the survival rate of the flies also increased after with the introduction of Yki and SD proteins. The above results indicate that the Hpo signaling pathway, which includes the Yki and SD proteins, plays an important role in regulating ISC proliferation and differentiation.

SW06.W32–13
Evolution of insect Midgut trehalases
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Trehalase is a widespread enzyme in insects, needed because their main circulating sugar is the disaccharide trehalose (glucose 2-1,1-glucose). Trehalase inhibition is harmful to insects, indicating that the enzyme is a good target for insect control. More information about insect trehalases could help the development of this field. Midgut transcriptomes (454 pyrosequencing and Illumina sequencing) obtained from 8 insects of 5 orders (Dicyoptera,
Hemiptera, Coleoptera, Diptera and Lepidoptera) revealed 11 trehalases: 4 membrane-bound and 7 soluble enzymes. Membrane-bound trehalases have a transmembrane helix near the C-terminal end. Amino acid sequence alignments of these sequences and others found in GenBank showed that Diptera trehalases have a region lacking 4 or 5 amino acids that on molecular modeling correspond to a surface random coil loop.

A cladogram of insect trehalases showed that the membrane-bound and soluble trehalases form two distinct branches, suggesting that trehalase gene duplication and divergence are prior to the separation of the paraneopteran and holometabolan orders. The exceptions are the Diptera trehalases that group together in the membrane-bound branch. Thus, it seems that among the dipteras the soluble trehalase was lost in evolution and it was regained when the trehalase gene for membrane-bound trehalase was duplicated and diverged. In all insects, soluble trehalase probably derived from the membrane-bound one by losing the C-terminal transmembrane loop.

Analysis of mRNA transcription by semi-quantitative reverse transcriptase-polymerase chain reaction showed that insect midgut membrane-bound trehalase is expressed also in other insect tissues, while some soluble trehalases (such as the one found in the Hemiptera Dysdercus peruvianus) are specifically present in the midgut.

The data suggest that the membrane-bound trehalase is responsible for the most basic functions, thus explaining why it is found in most insect tissues and was conserved along the evolution in all taxa examined.

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**SW06.W32–14 Xenobiotic-metabolizing system phase 1 in Opisthorchis felineus (Trematoda, Platyhelminthes)**

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Xenobiotic detoxification system is a biotransformation mechanism protects the body against xenobiotics and drugs. This ancient system is widely distributed in living organisms from bacteria to mammals. Nothing is known about this system in parasitic flatworms. Since biotransformation pathways are involved in drug resistance and could be new pharmaceutical targets, study of this system is a primary interest.

Human liver fluke *Opisthorchis felineus* infests bile ducts and the pancreas, has significant pathogenicity, and causes complications including liver cancer.

Aims of this study were (1) to identify cytochrome P450 (CYP450, CYP) coding genes in parasitic flatworms and (2) to test CYP activity in human liver fluke *Opisthorchis felineus*.

The only one cytochrome P450 has been identified in various species of parasitic flatworms including Trematoda (Opisthorchiidae, Schistosomatidae, Fasciolidae), Cestoda (Taeniidae).

We have cloned and sequenced CYP450 mRNA of *O. felineus*. The CYP amino acid sequence was reconstructed and confirmed by MS/MS. In contrast to the low level of amino acid sequence homology to other eukaryotic CYPs 3D model of the *O. felineus* CYP450 demonstrates high conformational similarity with mammalian CYP2 subfamily structures. The observed conformational similarity of *O. felineus* and human biotransformation proteins indicates a high probability of coincidence of their functions.

Indeed, we have demonstrated a monooxygenase CYP activity in *vivo* in fluke. It was similar to the mammalian CYP2E1 (HPLC) and CYP2B (MS) and was almost fully inhibited by specific inhibitor for CYP ketoconazole. The level of *O. felineus* CYP mRNA expression (Real-time PCR) in maritae (adult stage in definitive mammal host) was constitutive and significantly higher than in other life stages. So we have shown highly expressed functional monooxygenase system with broad substrate specificity and with conservative organization that may play an important role in parasite metabolic system.

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**SW06.W32–15 Research of the mechanisms of praziquantel action on fluke Opisthorchis felineus (Trematoda, Platyhelminthes)**

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Currently, praziquantel is the drug of choice against parasitosis caused by trematode infections. Praziquantel damages the permeability of cell membranes to calcium ions, damages the worm body surface, and causes the parasite death. However, mechanisms of its action and molecular mechanisms of praziquantel resistance are not known.

The aim was to identify molecular targets and phenotypic markers of praziquantel efficacy in trematoda *O. felineus*.

We found marked differences in sensitivity to praziquantel *ex vivo* in the fluke *O. felineus*. Praziquantel at 1 mg/kg/ml was ineffective against juvenile maritae, and, in contrast to published data on *C. sinensis*, did not result in death of *O. felineus* mature maritae. Using fluorescent staining assay, we have shown that the damage of the body surface and the death of *O. felineus* occurs only at praziquantel concentrations (100–500 mg/ml) many times higher than that in bile at therapeutic doses.

We investigated the cell depolarization of mature and juvenile *O. felineus* maritae in response to praziquantel. In addition, using Opisthorchiidae family nucleotides databases we identified the genes coding calcium ion membrane channels – molecular targets of praziquantel.

This work is supported in part by RFBR grant N 13-04-00662a and SB RAS Research Partnership grant N 19.

**SW06.W32–16 The fatty acids composition of Baikalian endemic amphipods Eulimnogammarus cyaneus Dyb. and Eulimnogammarus marituji Baz.**

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Lake Baikal is the one of the greatest freshwater lake in the world, containing roughly 20% of the world’s surface fresh water. It is the oldest and deepest lake. Lake Baikal is a unique nature object characterized by taxonomic diversity of inhabiting forms. Amphipods are the most abundant species group of Lake Baikal organisms. There are 344 endemic species of amphipods described in the lake.

The lipid and fatty acid compositions of freshwater amphipods are insufficiently studied. We studied fatty acid compositions of the Lake Baikal amphipods. Two endemic species have been studied: *Eulimnogammarus marituji* Baz. and *Eulimnogamm-
arcs cyanus Dyb. Methyl esters fatty acids have been prepared and analyzed by gas-liquid chromatography with mass spectrometric detection. The fatty acids composition has been denoted as per cent by weight of total acids content. The values have been given as mean±standard deviation (n = 3).

Contents of the main saturated acids: myristic acid (C14:0) was 5.03 ± 0.42% in E. marituji and 7.26 ± 0.62% in E. cyanus; palmitic acid (C16:0) – 20.77 ± 0.64% in E. marituji and 20.10 ± 0.67% in E. cyanus; stearic acid (C18:0)-2.38 ± 0.08% in E. marituji and 2.49 ± 0.24% in E. cyanus. The fatty acids were mostly unsaturated and their total amount was 71.82 ± 0.84% in E. marituji and 70.15 ± 1.17% in E. cyanus. The highest accounted content: oleic acid (C18:1n-9) was 25.58 ± 0.41% in E. marituji. 23.82 ± 0.59% in E. cyanus and eicosapentaenoic acid (C20:5n-3) 19.24 ± 1.0% in E. marituji, 18.26 ± 1.28% in E. cyanus. The content of other main fatty acids: palmitoleic acid (C16:1n-7) was 11.75 ± 0.62% in E. marituji and 12.19 ± 1.0% in E. cyanus; cis-vaccenic acid (C18:1n-7)-3.68 ± 0.10% in E. marituji and 3.36 ± 0.01% in E. cyanus; linoleic acid (C18:2n-6) 3.89 ± 0.27% in E. marituji and 4.35 ± 0.27% in E. cyanus; z-linolenic acid (C18:3n-3)-5.43 ± 0.22% in E. marituji and 4.48 ± 0.18% in E. cyanus; arachidonic acid (C20:4n-6)-2.25 ± 0.19% in E. marituji and 3.68 ± 0.43% in E. cyanus. Thus, the fatty acid composition in tissues of studied amphipod species was different, but includes a significant amount of essential eicosapentaenoic acid in both species.

**SW06.W32–17**

Monoacylglycerol lipase and fatty acid amide hydrolase are secreted from lysosomal and nonlysosomal sources in *Tetrahymena thermophila*

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In previous studies the two main degradative enzymes of the endocannabinoid system, the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase (MAGL), have been identified and characterized in the protozoan *Tetrahymena thermophila*. In this work the secretion of these enzymes was investigated. *Tetrahymena* has been regularly used as a model organism for the study of secretion and has two well characterized routes of secretion: the constitutive secretion of lysosomal enzymes and the regulated secretion of mucocysts. Initially, the secretion of both enzymes was investigated in starvation medium, using [3H]2-oleo-ylglycerol (2-OG) as substrate and in the presence or absence of FAAH specific inhibitor AM374. Both enzymes were secreted in the starvation medium, in a time dependent manner. The maximum secretion was ~5% after 4 h incubation and steadily declined afterwards. The secretion of these enzymes is a combined result of two different sources of constitutive secretion: a lysosomal and a non-lysosomal pathway. In the case of FAAH, the non-lysosomal pathway seems to be specific. Subsequently, stimulation of secretion with dibucaine leads to exocytosis of the mucocysts of *Tetrahymena*. In the isolated mucus MAGL and FAAH activity were determined, using [3H]2-OG as substrate in the presence of AM374 or MAGL specific inhibitor JZL184. In the case of MAGL, the activity measured was equivalent to that of the cell homogenate. Supernatant was enriched in FAAH activity, confirming our results about the existence of a non-lysosomal secretion pathway for this enzyme. Immunoblot analysis using anti-MAGL antibody revealed the presence of an immunoreactive protein at ~45 kDa in the mucus fraction, the supernatant and homogenate. Finally, confocal laser scanning microscopy (CLSM) showed mostly pericellular localization of MAGL, suggesting its existence in the mucocysts.

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**SW06.W32–18**

Cathepsin L-like peptidase from *Tribolium castaneum* larvae – a possible candidate for treatment of Celiac disease

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*Tribolium castaneum* is a model organism with the first sequenced genome among the order Coleoptera and one of the major pests of stored crops. The main food proteins of this insect are proline- and glutamine-rich wheat prolamins, and they are also present in the diet of most people. In 1% of the human population resistant to proteolysis prolamin peptides cause a hereditary disorder of the small intestine Celiac Disease. We have shown that the major digestive peptides in *T. castaneum* larvae are cysteine peptidases.

The aim of this study was isolation and characterization of the major digestive cysteine peptidase from *T. castaneum* larvae in view of its use for enzymatic treatment of CD. The enzyme was purified using gel chromatography and native PAGE. Post-electrophoretic activity was detected directly in the gel using original fluorographic substrate Glp-Phe-Ala-AMC. Using MALDI-TOF and MS/MS mass-spectrometry analysis the enzyme was identified as the most highly expressed in the gut cathepsin L (NP_001164001). Substrate specificity was studied using nine chromogenic and fluorographic substrates designed and synthesized in our laboratory, and two commercially available chromogenic substrates. In general, substrate specificity of *T. castaneum* digestive cathepsin L correlated with the specificity of human lysosomal cathepsin L. We also tested the effect of our enzyme on substrates containing fragments of immunogenic peptides of prolamin Abz-LPYPQQLPQ-EDDnp and Abz-QQFPFPQ-EDDnp. *T. castaneum* cathepsin L hydrolyzed these substrates two to four times faster than human cathepsin L. *T. castaneum* procathepsin L was expressed in *Pichia pastoris* expression system and the main characteristics of recombinant peptidase are compared to those of the native enzyme.

So, digestive cathepsin L from *T. castaneum* larvae seems quite promising in developing enzymatic medicine for treatment of CD.

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**SW06.W32–19**

Analysis of cysteine cathepsins in *Tenebrionidae*

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*Tenebrio molitor* and *Tribolium castaneum* are two closely related stored product pests (Coleoptera: Tenebrionidae) with similar
spatial organization of digestion and diet. The major role in their protein digestion belongs to cysteine peptidases (CPs) from C1 family, mostly cathepsins L and B, that have been discovered in mammals as lysosomal enzymes.

We explored diversity in gut CPs in *T. molitor* and *T. castaneum* larvae. Primary structures of *T. molitor* proteins were obtained from high-throughput Illumina sequencing of gut cDNA. *T. castaneum* sequences were obtained from *T. castaneum* genome [1]. Composition of substrate binding sites were derived by homology with human and animal CPs based on multiple sequence alignment and comparison of modeled and experimentally obtained 3D models. We also studied possible alternative localization of cathepsins to lysosomes or secretion to the midgut lumen using structural markers of mannose-6-phosphate pathway. Levels of genes expression were assessed by Illumina sequencing data for both insect larvae.

Seventeen sequences similar to cathepsins L and 15 to cathepsins B were found in *T. molitor* midgut. Nine of them were found in the previous paper [2], and 23 were new. From *T. castaneum* genome we used 15 cathepsin L-like sequences and 9 cathepsin B-like sequences [1]. *T. molitor* cathepsins L set contained 3 peptidases that showed binding substrates composition corresponding to human lysosomal cathepsin L. In *T. castaneum* we found only one typical cathepsin L peptidase. Composition of substrates in most peptidases from both organisms did not correspond to any of described cathepsin types. It is possible that there is a correlation between the level of expression and localization of cathepsins: high-expressed genes code secreted proteins and lysosomal enzymes are mostly coded by low-expressed genes.

This work was supported by RFBR (grants #12-04-01526-a) and ISTC (grant #3455).

References

**SW06.W32–20**

**Molecular and functional approaches for understanding cytochrome P450-based detoxification mechanisms in insect pests**

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The control of major agricultural pests and insects of public health, has been largely based on the use of insecticides. However, the pests evolve resistance to most chemicals rapidly, a phenomenon particularly striking in Mediterranean countries. By using a variety of approaches, including production and characterization of recombinant P450 proteins, in vivo ectopic expression in drosophila, and plant mediated RNA interference (RNAi), we try to understand the precise mechanisms involved in the detoxification process. We have recently successfully expressed a number of P450s from the major agricultural pests *Tetranychus urticae* and *Bemisia tabaci* that are implicated in resistance. For some of them, such as the bCyP6CM1 and the tuCyP392E10, we have verified that they are capable of catalyzing the detoxification of certain acaricides/insecticides (Karunker et al. 2009, Nauen et al. 2013, Demaeght et al. 2013), and/or to interact strongly with certain groups of active ingredients in IC50 kinetic assays. We also showed that the transgenic expression of the aCYP9J28 from pyrethroid resistant *Aedes aegypti* mosquitoes confers high levels of pyrethroid resistance in drosophila. We have finally developed transgenic plants, targeting the P450 detoxification pathways, aiming to investigate the role of P450-based detoxification in the ability of *T. urticae* and *B. tabaci* to overcome insecticide toxicity as well as to cope with plant allelochemicals.

References

**SW06.W32–21**

**Membrane unsaturation contributes to stress resistance and longevity of wild-type and long-lived mutant strains of *Drosophila melanogaster***

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Available evidences suggest that long-lived species evolved by reducing the relative abundance of those structural components that are highly susceptible to oxidative damage, thus conferring to the cellular constituents a higher structural stability and lower susceptibility to oxidative damage, along with low rate of mitochondrial free radical generation and oxidative molecular damage. This intrinsically high resistance to modification that likely contributes to their superior longevity is obtained in the case of lipids by decreasing the degree of fatty acid unsaturation. In this context, the aim of the present work was to study, by first time, the degree of membrane unsaturation and specific markers of protein damage of long-lived wild-type and mutant strains of *Drosophila melanogaster*. With those purposes, we used highly sensitive gas chromatography/mass spectrometry methods. We studied membrane unsaturation in i) three independent wild-type strains of *D. melanogaster* which show a substantial variation in longevity, and ii) a new transgenic *Drosophila* model expressing the yeast NADH dehydrogenase Ndi1 which confers increased longevity. Regression analysis between lipid parameters and longevity of wild-type and mutant strains of *Drosophila melanogaster* shows that the transgenic expression of the aaCYP9J28 from pyrethroid resistant *Aedes aegypti* mosquitoes confers high levels of pyrethroid resistance in drosophila. We have finally developed transgenic plants, targeting the P450 detoxification pathways, aiming to investigate the role of P450-based detoxification in the ability of *T. urticae* and *B. tabaci* to overcome insecticide toxicity as well as to cope with plant allelochemicals.
lipid resistance to the oxidative damage in long-lived species. Globally, all these comparisons (i) support an important role for membrane fatty acid composition in the determination of longevity, and (ii) reinforce the idea that the connection between membrane unsaturation and longevity is not restricted to vertebrates.

**SW06.W33 Bioengineering: Fundamentals and Application (VI-W33)**

**SW06.W33-1**

**Biocatalysis and metabolite synthesis**

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Biocatalysis is one of nature’s key strategies for the synthesis of metabolites required at the right place and time by biological cells or compartments where they are synthesized or where they are transported to. Biosynthesis in nature makes use of a system of highly selective biocatalytic reactions transforming metabolites along reaction pathways with high performance and high molecular economy. The architecture of these pathways within the cellular reaction space of the cell or a smaller unit provides a blueprint and inspiration for the utilization of biocatalysis in synthetic routes to metabolites using either isolated enzymes or whole cells. The combination of retrosynthetic analysis with the rapidly growing enzyme data is used as starting point for developing novel enzymes and novel synthetic applications of existing enzymes. The synthesis and purification of a variety of metabolites can be significantly reduced by using enzyme-catalyzed reactions. The search of enzymes for reaction steps posing problems, e.g. due to lengthy reaction sequences, safety, health and environment issues or are unknown, is particularly useful. Recent progress and discoveries and applications of biocatalysis in the synthesis of central and remote metabolites will be discussed.

**SW06.W33-2**

**Understanding structure–function relationship in protein families: bioinformatics and molecular modeling provide new concept for enzyme engineering**

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Analysis of sequence and structure information in enzyme families provide opportunity to rationalize protein engineering and move away from unguided evolutionary stochastic approaches. Homologous enzymes evolved from a common ancestor to retain a general function but diverged in more specific features and can be divided into subfamilies with different functional properties such as specificity, enantioselectivity, stability, etc. Not all positions can be subjected to mutations as some residues are crucial to maintain structure and function and thus may be constrained in the allowed residue types. Conserved positions can define general properties of the entire family (for example, have direct roles in enzyme catalytic machinery) but they do not explain functional diversity.

New method of bioinformatic analysis has been developed [1–3] to identify subfamily-specific positions (SSPs) – conserved only within protein subfamilies, but different between subfamilies – that seem to play important role in functional discrimination, and used to study how lipase and amidase catalytic activities are implemented into the alpha-beta hydrolase fold. Subfamily-specific positions of α/β-hydrolases with lipase and protease activities were identified and used as hotspots to introduce amidase activity into *Candida antarctica* lipase B (CALB). Molecular modeling was applied to evaluate influence of selected residues on binding and catalytic conversion of amide substrate by corresponding in silico library of mutants. In silico screening was implemented to select reactive enzyme-substrate complexes that satisfy knowledge-based criteria of amidase catalytic activity. Selected CALB variants were produced and showed significant improvement of experimentally measured amidase activity [4].

Developed method was also applied to study evolution of structure-functional relationship in other enzyme families: Ntn-hydrolases, penicillin-binding proteins, etc. It was shown, that patterns of SSPs can be effectively used to design enzyme mutants with improved functional properties. Based on these results, we suggest that bioinformatic analysis of subfamily-specific positions can be implemented in the laboratory practice to study structure-function relationship and for rational design of advanced biocatalysts.

**References**


**SW06.W33-3**

**Sustainable technologies for new, biobased value chains**

M. Wubben  
*CTO, Royal DSM, The Netherlands*

Today’s market needs are driven by a number of major global trends and challenges. At DSM we are using our innovative strengths to address some of the most important trends and challenges, such as climate change, increasing energy scarcity, over-stretched healthcare systems and hidden hunger.

In over a century, DSM has moved from coal via fertilizers, petrochemicals and plastics to become a leading Life Sciences and Materials Sciences company. We strive to find effective, sustainable solutions for the issues facing the world today and tomorrow in Health, Nutrition and Materials. Innovation requires the focused and smart collaboration of all relevant disciplines, from R&D and sourcing, to manufacturing and marketing and sales.

DSM has developed green technology solutions by combining our technological competences in Chemistry, Biotechnology and Materials Sciences. We have created technologies for conversion of renewable, agro-based feedstocks to bio-fuels and bio-based performance materials that are helping the world switch from a fossil feedstock-dependent economy to a sustainable bio-based one.

We strongly believe in Open Innovation. The magnitude of societies’ challenges, the complexity of current day solutions as well as the urgency to create these, ask for close collaboration and partnerships between industry, academia and other organizations in our society. We strongly believe in those partnerships...
and exploit them fully as is illustrated for instance in our joint ventures with POET and Roquette. In addition, DSM is actively participating in a shared Biorefinery Pilot Plant in Delft and in various running and upcoming PPP’s such as BRIDGE, the Bio-based PPP in Horizon 2020.

The presentation will provide some illustrative examples of DSM’s activities in the area of Green Chemistry and Biorefineries.

**SW06.W33–4**

The bioeconomy asks for cutting-edge Science and Technology

M. Kircher

CLIB2021 – Cluster industrielle Biotechnologie, Düsseldorf, Germany

The unfolding bioeconomy offers growing business options in renewable feedstock, biotechnological processes and products as well as instruments, machinery and production equipment. The success of all these business sectors will be based on cutting-edge science and technology. Top science and targeted research and development are therefore most critical success factors.

The broad use of biomass on industrial scale asks for plant breeding, biomass harvesting, logistics and storage, processing and transformation and last not least methods to link and integrate biotechnological and chemical processes.

This presentation will discuss fields of science to be prioritized with as special focus on biotechnological processes from an industrial view.

**Curriculum Vitae:**

Dr. Manfred Kircher

Dr. Kircher brings along more than 30 years of experience in the field of investments, biotechnology, R & D, production and project development. He joined Degussa in 1981 and was placed in charge of microbial strain development. Since 1993 he co-built Femas, an international joint venture for amino acid production in Slovakia and from 2001 to 2004 he managed Degussa’s investment in Burrill & Company - a San Francisco based venture capital bank. Later he served in Evonik Industries as vice-president biotechnology partnering and branding. In parallel he has build the Bioeconomy Cluster CLIB2021 as chairman of the board since 2007.

Dr. Kircher has been awarded with an honorary professorship of the Michurinsk State Agrarian University (Russia).

**SW06.W33–5**

Laccases: blue enzymes for green chemistry

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Laccases are oxidoreductases belonging to the multinuclear copper-containing oxidases. The overall outcome of their catalytic cycle is the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to give four radicals.1

Typical substrates of laccases are phenols and aliphatic or aromatic amines, the reaction products being mixtures of dimers or oligomers derived by the coupling of the reactive radical intermediates. For instance, these biotransformations have been exploited to isolate new dimeric derivatives of natural phenolic molecules (resveratrol and its analogues, β-estradiol, totarol, silybin).2 In these studies a significant influence of the solvent on the reaction outcomes has also been observed.3

More recently, we have described the use of laccase-catalyzed reactions for the selective hydroxylation of ergot alkaloids and for the synthesis of the bisindole alkaloid anhydrovinblastine.4

Additionally, laccases oxidation of non-phenolic groups, particularly benzyl and – more generally – primary alcohols, is also possible thanks to the ancillary action of the so-called “mediators” (i.e., TEMPO, HBT, ABTS): the oxidation step is performed by the oxidized form of a suitable mediator, generated by its interaction with the laccase. Accordingly, a series of sugar derivatives and of natural glycosides have been oxidized.5

**References**


**SW06.W33–6**

Biophotonic applications in molecular medicine

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We are applying high-performance fluorescence microscopy and other biophotonic techniques to better understand and characterize processes such as HIV transmission and cellular autophagy. We hope these discoveries will lead to important breakthroughs in molecular medicine.

The advent of biotechnology including polymerase chain reaction (PCR), gel electrophoresis, immunoassays, DNA/protein microarrays, and bioinformatics – have enabled medical scientists to make tremendous progress in understanding the molecular basis of human health and disease. Yet, despite the breakthroughs of genomics and proteomics, major challenges remain to be overcome. The relative lack of tools and methods to visualize and manipulate living biological systems at the subcellular and molecular level has become a major obstacle to further progress in areas such as cancer biology and medicine, neuroscience, cardiovascular disease, infectious disease, and stem cell research/regenerative medicine. We introduce two examples of ongoing research at CBST to better understand molecular mechanisms in cancer biology and infectious disease, respectively:

Quantification of Cellular Autophagy using 4D Image-based Cytometry [1]. Autophagy, or programmed cell recycling, is a process that occurs when cells undergo nutritional starvation. Based on previous research which shows that autophagy plays an
important role in modulating breast and prostate tumor cell killing by arginine demininase (ADI) – we used deconvolution fluorescence microscopy to obtain 3D image sequences, from which we could extract statistical information about the number, distribution, and degree of colocalization or fusion between autophagosomes and lysosomes. This information not only helps us to understand the basic relationship between autophagy and apoptosis, but for the discovery of new cancer pharmaceuticals may allow us to compare the relative efficacy of other candidate chemotherapy drugs that also act through autophagy.

Endocytic Mechanism of HIV Transfer between T Lymphocytes [2]. The global AIDS epidemic and continued failure (despite international efforts) to develop an effective vaccine against HIV strongly suggests that the basic mechanisms of viral infection and proliferation are still not completely understood. Utilizing a competent, highly-fluorescent modified clone of HIV developed by colleagues at the Mount Sinai School of Medicine in New York, we performed high-speed spinning disk 3D scanning confocal microscopy of live infected T cells to visualize for the first time ever, the effective transmission of HIV through direct cell contact (rather than by conventional cell-free mechanisms) Our observations support a model in which HIV gains entry into uninfected cells through an endocytic mechanism, and the measurements we have obtained may be useful for analyzing the effects of drugs meant to inhibit HIV infection and transmission.

References

SW06.W33–7
Microscopic analysis of cell death by metabolic stress-induced autophagy in prostate cancer
C. A. Changou1,2,3, R. Holland Cheng1, R. Bold2, H.-J. Kung1,3,4 and F. Y. S. Chuang1,3
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Autophagy promotes cellular survival against environmental stress and nutritional starvation. We have recently shown that some prostate cancers undergo metabolic stress and caspase-independent cell death following exposure to arginine demininase (ADI, an enzyme that degrades arginine in tissue). The aims of our current investigation into the application of ADI as a novel therapy are to identify the components mediating tumor cell death, and to determine the role of autophagy (stimulated by ADI and/or rapamycin) on cell death. Using advanced fluorescence microscopy techniques including 3D deconvolution and structured-illumination superresolution imaging, we show that prostate tumor cells treated with ADI for extended periods, die exhibiting a morphology that is distinct from caspase-dependent apoptosis; and that autophagosomes forming as a result of ADI stimulation contain DAPI-stained nuclear material. Fluorescence imaging (as well as cryo-electron microscopy) show a breakdown of both the inner and outer nuclear membranes at the interface between the cell nucleus and aggregated autophagolysosomes. Finally, the addition of N-acetyl cysteine (or NAC, a scavenger for reactive oxygen species) effectively abolishes the appearance of autophagolysosomes containing nuclear material. We hope to continue this research to understand the processes that govern the survival or death of these tumor cells, in order to develop methods to improve the efficacy of cancer pharmacotherapy.

SW06.W33–8
Unusual fluorescent proteins: design and applications
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 GFP and other fluorescent proteins became a routine instrument of analysis of live cells and organisms which is used in thousands of laboratories every day. However, in addition to such robust applications, there is a room for advanced sophisticated models where fluorescent proteins with “unusual” spectral or biochemical properties are in demand.

Known natural diversity of GFP-like protein family provides a rich source of new fluorescent proteins from marine animals. Besides, directed molecular evolution is successfully used to create variants of fluorescent proteins never encountered in nature. For example, we developed KillerRed – phototoxic fluorescent proteins capable of light-induced production of reactive oxygen species (ROS). Importantly, KillerRed allows light-induced ROS production to be strictly localized to different compartments and subcompartments within a cell. It was shown that KillerRed-mediated oxidative stress at different locations results in clearly different molecular events and cell responses.

Recently, we designed CFP variant with an anionic tryptophan-based chromophore. In contrast to green and red fluorescent proteins usually containing anionic tyrosine-based chromophores, no charged states of the CFP chromophore have been described before. We believe that switching between protonated and deprotonated tryptophan in chromophores of fluorescent proteins represents a new unexplored strategy to control their spectral properties.

Unexpected ability of regular GFPs to act as light-induced electron donors in photochemical reactions with biological electron acceptors has been discovered recently. These reactions have important consequences for practical applications of fluorescent proteins as well as for our understanding of evolution and biology of this amazing protein family.

SW06.W33–9
Computation-supported enzyme engineering for preparative biocatalysis
Biotransformation and Biocatalysis, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, the Netherlands

Many processes in industrial biocatalysis rely on microbial enzymes that play a role in biodegradation in their natural host. The use of such enzymes for preparative conversions usually requires improvement of their biochemical properties, such as thermostability, solvent tolerance, substrate range, and chemo-, regio- or stereoselectivity. An established protein engineering method is directed evolution, which is based on (semi-)random mutagenesis of gene encoding the target enzyme, followed by
extensive high-throughput screening. We have investigated the integration of computational methods in library design strategies in order to more efficiently sample sequence space for enzymes with improved properties. For thermostabilisation, we explored a strategy in which point mutants and disulfide bond mutants are first generated in silico and evaluated by calculating differences in free energy of folding (ΔΔGfold). Only variants that passed the computational test were expressed and tested, and the experimentally confirmed beneficial mutations were combined, again after testing multiple combinations by molecular dynamics simulations. This allowed spectacular thermostabilisation of an epoxide hydrolase in only three rounds of evolution.

A second way to reduce laboratory screening is the use of molecular dynamics simulations to identify sites in a protein where functional motions take place, and restrict mutations to these sites. For example, we used molecular dynamics to explore the opening of a loop region in phenylalanine aminomutase, which is required for substrate binding and release. Mutants constructed on the basis of the simulation results were tested experimentally, and it appeared that substitutions that influence loop opening can be used to alter the lyase:mutase ratio, thereby converting the mutase to an ammonia lyase, and to increase the catalytic rate.

These and other examples illustrate that the use of MD simulations and integration of computational protein design can strongly improve library quality in directed evolution and reduce the screening effort required for improving enzyme properties. This is especially important for enzymes that cannot be expressed or measured in high-throughput format.

References


SW06.W33–10
Live optical imaging of mammalian embryos to assess congenital diseases
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Every year, about 6% of infants worldwide are born with serious birth defects. Moreover, birth defects are the leading cause of infant deaths. Despite of the major effort in the biomedical community to understand the nature of congenital defects, 70% of the causes of birth defects remain unknown. Mouse models of human diseases provide a powerful resource for exploration of molecular regulation of development and pre-clinical studies. A live high-resolution imaging method for mouse embryos would contribute to high-throughput morphological screening of mouse mutants with developmental abnormalities. Recently we have developed methods for live mouse embryonic imaging in static culture on the imaging stage with Confocal Microscopy and Optical Coherence Tomography (OCT). This approach allows to image cardiodynamics and hemodynamics at the embryonic stages E8.5–E16.5. Our results demonstrate that live optical imaging using endogenous and exogenous contrast agents is a potentially useful tool to study cardiac function in mouse models with heart defects.

SW06.W33–11
Quantum based simulations of structure and spectra of photoreceptor proteins
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Studies of photoreceptor proteins involved in the sensing and response to light in a variety of organisms are of great importance to understand the atomic details of the dynamics in proteins required for their functioning. The successful design of these proteins with improved properties requires experimental realization; however, computational chemistry is a useful tool, e.g., for testing the effect of key mutations on the optical properties of photoreceptor proteins in order to provide the initial structural motif for experimental studies. We discuss recent results of computer simulations based on the quantum theory carried out for proteins with different chromophores. Atomic structures on the ground and excited electronic states as well as optical spectra in the model systems are generally considered within the quantum mechanical – molecular mechanical approaches.

In particular, we predict that excitation of the kindling fluorescent protein, the Ala143Gly variant of the natural chromoprotein asFP595, may result in fluorescence from the cationic form of the chromophore which is unusual for the members of the green fluorescent protein (GFP) family. We also show that the Ala143Gly mutation in asFP595 noticeably increases lifetime of fluorescence state due to the increased free energy barrier for the cis-trans ground state isomerization reaction.

As another example, we computationally constructed GFP variants in which the conventional anionic GFP chromophore, 4-hydroxybenzylidene-imidazoline, is sandwiched between the two tyrosine residues resulting in a “triple-decker” motif. The emission band from conformations with the trans cationic chromophore should be noticeably shifted to the blue side around 520 nm compared to the well-known red fluorescence around 600 nm arising from the cis anionic species.

Since no reliable quantum based theoretical models describing the energy landscape along the assumed proton transfer route A→I→B in GFP have been reported so far we characterize computationally the structures and optical spectra of the corresponding forms.

SW06.W33–12
Bioinformatic analysis of penicillin-binding proteins identified amino acid residues responsible for substrate specificity of D-aminopeptidase from Ochrobactrum anthropi
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D-aminopeptidase from Ochrobactrum anthropi (DAP) is a unique member of penicillin-binding proteins (PBP) family that converts mainly D-alanine containing peptides, amides, and esters with high D-stereospecificity. Consequently, in this work we aimed to study structure-function relationship of substrate recog-
tion in DAP and rationally design its specificity toward substrates with bulky side-chain residues – D-phenylalanine and D-leucine derivatives.

Recently developed bioinformatic analysis [1,2] of penicillin-binding proteins (PBP) family was used to identify subfamily-specific positions (SSPs) that are conserved within functional subfamilies of PBP, but different between subfamilies and therefore expected to be responsible for functional diversity among homologs. Specific positions that participate in formation of acyl group binding subsite were selected as hotspots to rationally design specificity of DAP toward selected substrates by introducing amino acid types observed in other subfamilies. Molecular modeling was used to screen the corresponding in silico library of DAP mutants and select the best variants by evaluating their ability to stabilize near-to-attack conformation of the substrate in the active site cleft. Selected triple mutant was purified and characterized experimentally to successfully hydrolyze aromatic and branched aliphatic D-amino acid derivatives in a full agreement with computational predictions.

The results outline perspectives for bioinformatic analysis of subfamily-specific positions as a systematic tool to understand structure-function relationship in enzymes. Selection of subfamily-specific positions as hotspots for rational design of enzyme properties is suggested as a new protein engineering strategy.

References

SW06.W33–13
3rd generation fluorescent proteins with enhanced properties for FRET and for monitoring signaling in living cells
Section of Molecular Cytology & van Leeuwenhoek Centre for Advanced Microscopy, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands

Since the cloning of the green fluorescent protein from Aequoria victoria, numerous fluorescence microscopy applications have been described in the literature employing new GFP variants. Here we report a screening method that, in addition to fluorescence intensity, quantifies the excited state lifetime of a fluorescent protein, providing a direct measure for the quantum yield of the fluorescent protein. The novel approach was used to screen a library of cyan fluorescent protein (CFP) variants and in combination with structure-guided design yielded mTurquoise2, a very bright cyan fluorescent protein variant. mTurquoise2 has a marked increased quantum yield of 0.93 (a record for monomeric fluorescent proteins), and a seriously increased (single-exponential) lifetime of 4 ns. It is the preferred donor to YFP variants (like mVenus or mCitrine) with an R0 of 5.83 nm. More recently, with the lifetime screening approach, also serious enhanced red fluorescent proteins were produced displaying fluorescence lifetimes above 3 ns, i.e. more than two-fold increased fluorescence lifetime as compared to mCherry.

We apply the different probes for the study of GPCR-triggered signaling across the membrane in single mammalian cells. Using mTurquoise, a new Gq FRET sensor was developed reporting on activation of the G protein by endogenous expressed receptors. Also downstream signaling events like multiparametric imaging of lipid-derived second messengers, PtdInsP2-dependent PLC relocalization, and RhoGEF activation using a variety of FRET, ratio imaging- and TIRF-microscopic applications will be presented.

References

SW06.W33–14
Bioengineering tools in antibody humanization
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Humanization of antibody variable domains is a necessary step for the conversion of murine monoclonal antibodies into potential therapeutic agents. The majority of humanized antibodies after primary grafting of murine CDRs onto human frameworks have decreased affinity compared to their chimeric counterparts. This phenomenon is accounted for by the influence of human frameworks on conformation of the antigen-binding site. The paratope conformation can be restored by introduction of one to several murine amino acid residues (back or reverse mutations) into the key positions of human frameworks. The success of such substitution depends on the understanding or assumption of the role of each particular framework amino acid residue in maintaining and stabilizing tertiary structure of antibodies.

Although the prominent framework residues responsible for supporting an antigen-binding site, such as “canonical residues” and residues in Vernier zones, are known from the antibody amino acid sequence, the more thoroughful structural analysis can be undertaken by providing adequate computational model of antibody.

For the humanization of an anti-interferon-gamma antibody and the antibody to a component of B. anthracis exotoxin the 3D structural models of the light and heavy chain variable domains were built and optimized using molecular dynamics (MD) algorithm. The MD simulation was estimated using Gromacs software (http://www.gromacs.org/). In addition, the sur-
face hydrophobicity patterns were obtained using Platinum software (http://model.nmr.ru/platinum/). The models obtained were used for the assessment of the influence of possible reverse mutations in framework regions on the conformation of the antigen-binding site. The mutagenized Fab fragments of the humanized antibodies carrying selected reverse mutations were expressed in E. coli cells. It was shown that the introduction of reverse mutations enabled us to restore the affinity and improve the biological activity of the humanized antibodies.

SW06.W33–15
Microbial production of amino acids: perspective approaches in metabolic engineering
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Practically-oriented metabolic engineering includes a number of approaches based on fundamental knowledge about mechanisms of a living cell functioning. Several important milestones in the development of metabolic engineering could be noted. Among them, (i) traditional selection; (ii) direct modification of specific metabolic pathways based on precise genetic engineering manipulations with a microbe chromosome, supported by systems biology achievements such as -omics technology, artificial metabolic control, laboratory adaptive evolution, etc.; (iii) opening up a new era of metabolic engineering due to the last progress of synthetic biology enabling to expand a range of hosts, substrates, metabolic pathways and, thereby, to expand a range of natural and unnatural compounds that could be produced microbiologically.

In this work, with the help of the above approaches, the bacteria, producing L-amino acids, were designed. Modification of key enzymes was performed by traditional selection or by site-directed mutagenesis on the basis of structural biology and bioinformatics data. This step was followed by intra-chromosomal amplification and modulation of the expression of a lot of genes of interest. To this end, we applied a number of genetic engineering methodologies, widely used for this purpose as well as specially adapted or newly developed on the basis of bacteriophages recombination systems. These methodologies enabled precise modifications of the chromosome of industrial microorganisms, including (i) alteration of structural and/or regulatory regions of biosynthetic genes; (ii) inactivation or attenuation of genes that control undesirable by-products synthesis; (iii) introduction of heterologous DNA, natural or de novo synthesized, responsible for effective conversion of a substrate into target compound due to alteration of substrate (cofactor) specificity or realization of novel metabolic pathways; (iv) optimization of the expression of genes essential for amino acid export.

SW06.W33–16
Enzymatic way to modify oligosaccharides
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Glycoside hydrolases (glycosidases, E.C. 3.2.1.-) are key players in degradation of polysaccharides and one of the most used and studied groups of enzymes. The enzymes catalyze cleavage of glycosidic linkages between two saccharide residues or between glyconic and aglyconic components. The elucidation of the myriad glycan structures of glycochemistry creates a need for methods of synthesis of saccharides, both for the assessment of function and for biotechnological purposes. Transglycosylation by wild-type and genetically modified retaining glycohydrolases still has its place in the glycochemist’s armamentarium, because of the disadvantages of the alternatives.

During last decade, our laboratory has been focusing on search, isolation and detailed studies of structural and functional properties of a number of glycoside hydrolases from fungal and bacterial origins. Combination of state-of-the art biochemical, biophysical, computational and genetic methods enable us to shed light on the mechanism of catalysis of several enzymes at the molecular level, modify and efficiently use them in chemoenzymatic synthesis of new substrates and carbohydrate-containing compounds. Structure and functional studies of various glycoside hydrolases revealed mechanisms of action of alpha-galactosidase from Thermotoga reesei (Golubev et al., J. Mol. Biol., 2004, 339:413–422), beta-galactosidase from Penicillium sp. (Rojas et al., J. Mol. Biol., 2004 343:1281–1292) and exo-inulase from Aspergillus awamori (Arand et al., Biochem. J., 2002, 362: 131–135; Kulminskaya et al., Biochim. Biophys. Acta, 2003, 1650: 22–29), resulted in definition of the architecture of the active sites and identification of catalytically active amino acid residues. Several beta-mannooligosaccharides modified with chromophoric groups were synthesized within transglycosylation reactions by beta-mannosidase from Thermotoga reesei. Unique feature of A. awamori beta-xilosidase to catalyze synthesis of modified and non-modified xylooligosaccharides with d.p. of more than 10 was thoroughly investigated and a model of active center architecture was presented. It suggests existence of additional sites for binding for donor and acceptor that take part in the reaction of transglycosylation. Minimal kinetic scheme describing this mechanism was postulated. Proposed mechanism of the reaction was confirmed by experimental results with the reaction rate measurements and effects of addition of external nucleophile (methyl-beta-xylopyranoside) (Eneysskaya et al., Arch. Biochem. Biophys., 2007, 457:225–234).

Detailed biochemical studies of alpha-galactosidases from clan D (GHF 27 and 36) confirmed the homology of the active sites structures and similar mechanism of action (Comfort et al., Biochemistry, 2007 46:3319–3330). We found that alpha-galactosidase from Thermotoga maritima is suitable for the syntheses of valuable galacto-oligosides. This enzyme is known to possess transglycosylating activity and capable of working at elevated temperatures. Single amino acid substitutions predicted to change the transglycosylation patterns of a GH 36 alpha-galactosidase were identified on the basis of molecular modeling. The covalent enzyme-galactosyl intermediate was constructed in silico for each mutant, and interactions with possible acceptors calculated. On the basis of simple assumptions, residues were identified whose mutation could yield increased regioselectivity of transglycosylation. These mutations were then made, and some of them indeed produced specifically-linked galactosides.
SW06.W33–17
Bioconversion of airborne methylamine by immobilized methylamine oxidase from Hansenula polymorpha

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Methylamine (MA) is a toxic pollutant that has a negative effect on human health when present in indoor air and therefore it should be removed by ventilation or by conversion into non-toxic products. Methylamine oxidase (AMO), a copper-containing Hansenula polymorpha enzyme, was isolated from a recombinant strain of the yeast Saccharomyces cerevisiae overexpressing the gene HgAMO. This enzyme was tested for its ability to oxidize airborne MA. A continuous fluidized bed bioreactor (CFBR) was designed to enable bioconversion of airborne MA by AMO immobilized in calcium alginate beads. The results demonstrated that the bioreactor with immobilized AMO provided almost total elimination of airborne MA. Since the formaldehyde (FA) was formed as a product of the MA oxidation, a two-step bioconversion scheme for elimination of both MA and FA was designed. This scheme included two CFBRs in series, containing enzymes immobilized in calcium alginate: the first reactor contained AMO and the second one contained alcohol oxidase (AOX) from the yeast H. polymorpha. In the first step, airborne MA was converted into FA and in the second step, the FA was oxidized into formate. Almost 100% elimination of airborne MA and FA was achieved in the two-step system, providing outlet concentrations of MA and of FA that are 10 times lower than their threshold limit values.

SW06.W33–18
Engineering human cytochrome P450 4F11 and omega-hydroxylation of fatty acids

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Cytochrome P450 enzymes are a versatile class of monooxygenase proteins able to make at least 40 different types of reactions such as reduction, desaturation, ester cleavage and hydroxylation.

In particular, α-hydroxylation of fatty acids represents the most difficult reaction catalyzed by these enzymes in terms of the regio- and stereoselective oxidation of unactivated hydrocarbon C–H bonds to the corresponding hydroxyl (C–OH).

Here the gene encoding human P450 4F11 was cloned in pCW vector and successfully expressed in E. coli leading to 10 mg of pure protein per litre of culture. Furthermore, a chimeric protein (P450 4F11/BMR) consisting of P450 4F11 and the soluble reductase domain of P450 102A1 (Bacillus megaterium-BMR) was engineered using ‘molecular Lego’ approach [1]. The catalytic self-sufficiency of this protein would greatly simplify the in vitro activity studies of P450 4F11.

Both the enzymes were spectrophotometrically characterized. The P450 4F11 presented an atypical CO binding spectrum which is related to the covalently bound haem which is shown here for the first time for this isoform.

Palmitic and oleic acids were chosen as substrates to compare P450 4F11 and P450 4F11/BMR activity. The products were characterized as α-hydroxylated fatty acids by gas chromatography. The analysis of the reactions confirmed that the fatty acids used are substrates oxidized by both the proteins tested. Moreover, the activity of the engineered self-sufficient chimera was found to be comparable to that of the wild type P450 4F11.
Methods development for high level recombinant factor IX production in mammalian cells
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Goal and scope: Human coagulation factor IX is a major therapeutic for the treatment of hemophilia B. The goal of the present study was the development of methods for generation of highly productive factor IX secreting cell lines with stable secretion levels of functionally active factor IX.

Results: A specialized plasmid vector p1.1 containing fragments of flanking regions of translation elongation factor 1 alpha gene of Chinese hamster and dihydrofolate reductase as a selection marker was used for factor IX cDNA expression. For obtaining of polyclonal populations of stably transfected cells with the maximum level of factor IX secretion the selective culture medium with the addition of methotrexate was used. It was found that the level of secretion of factor IX is increasing with an increase in the concentration of methotrexate, and the time for stably transfected cell population generation remains virtually unchanged. In the course of clonal factor IX producer cell lines generation by limited dilution from the polyclonal population it was discovered that highly productive clones represent more than 10% of total screened clonal lines amount. The concentration of secreted factor IX for these clones ranged 2–5.4 IU/ml for confluent adhesive cultures.

Monoclonal antibodies to factor IX propeptide were generated using synthetic peptide-KLH conjugate for mice immunization. Purified monoclonal antibodies were used for monitoring of propeptide processing level in CHO-producer cells by specific ELISA assay.

Conclusions: High level factor IX producer cell lines can be obtained without amplification of the target gene stages using the plasmid vector containing the translation elongation factor 1 alpha of Chinese hamster gene fragments, level of the propeptide processing can be measured by specific ELISA assay using monoclonal antibodies to FIX propeptide.

Purification and characterization of Prunus cerasifera HNL and its application for enantiomeric synthesis of cyanohydrins
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Hydroxynitrile lyases (HNLs) serve the cleavage and the synthesis of optically active (chiral) cyanohydrins—a carboligation process. HNLs are widely used to synthesis many industrial products such as α-hydroxy aldehyde and ketones, β-amino alcohols, α-fluoro cyanides e.t.c. [1]. The cleavage of the cyanohydrins by the plant-a cyanogenesis process—serves as a defence strategy against herbivores and other microorganisms by liberating toxic hydrocyanic acid (HCN) from cyanogenic glycosides [2].

In this study, HNL was purified and characterized from the seeds of Prunus cerasifera fruits which have not been used as HNL sources up to now purified 14-fold with 26% yield by chromatography on hydrophobic interaction chromatography and size exclusion chromatography. The estimated molecular weight of purified HNL was found to be 110 kDa. The optimum pH, temperature, Kₘ and Vₘₐₓ values were determined as 6.0, 25°C, 0.31 mM and 174 U/mg prot., respectively for lyase activity of PcHNL.

The enzyme was also used to investigate the application for the synthesis of (R)-mandelonitrile, (R)-2-chloromandelonitrile is a precursor for (R)-2-chloromandelic acid which is a key intermediate for a potent oral anticoagulant, which reduces the risk of cardiovascular events and (R)-2-hydroxy-4-phenylbutyronitrile that serves as important synthetic intermediate for the production of nonsulfonyl angiotensin-converting enzyme (ACE) inhibitors referred to as ‘prils’.

In conclusion, the new enzyme PcHNL can be used for synthesis of mentioned cyanohydrins with a higher yield and enantioselectivity.

References

Immobilization of Aspergillus niger EH onto modified Eupergit C and its application for asymmetric hydrolysis of (R/S)-styrene oxide
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Enantiopure epoxides and their corresponding diols are key synths in the preparation of beta-adrenergic receptor blocking agents, nematicides, anticancer and anti-obesity drugs [1,2]. Due to the increasing demand for these chiral compounds with sustainable methods, enzyme-catalyzed applications biocatalytic methods have been applied have been developed to produce such intermediates in enantiopure form [3]. Epoxide hydrolases (EHs, EC 3.3.2.3) catalyze addition of a water molecule to oxirane ring leading to formation of corresponding vicinal diol. These enzymes are do not require any cofactors for their activities, found in all living organisms and exhibit high regio-, stereo- and enantio-selectivity towards a numerous of substrates [4]. However, their limited long-term operational stability and relatively high cost have hampered commercial applications of EHs. Therefore, this study aims to prepare robust EH preparation by immobilizing Aspergillus niger EH onto modified Eupergit C and to apply immobilized EH preparation for asymmetric hydrolysis of (R/S)-styrene oxide.

The results showed that (S)-styrene oxide yield was obtained with 48% yield after 240 min reaction time and its enantiomeric excess (ee) value was determined as >99%. Meanwhile, the ee value of (R)-1-phenyl-1,2-ethanediol was found as 92.3%. Enantioselectivity of EH was enhanced 1.9-fold towards styrene oxide upon the immobilization.

References

Abstracts
SW06.W33–23

Light dependent activity of restriction endonucleases

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Sequence-specific endonucleases with extended recognition sites can cleave a unique site in complex genomes. Since these nucleases can show off-target cleavage activity, spatio-temporal control of their activity is necessary for the precise genome engineering. It would be desirable to control the enzymatic activity by an external signal, e.g. by light. Such photoregulation is based on the azobenzene ‘photoswitch’ [1]. Azobenzene isomerizes between the extended trans- and cis-configuration by illumination with UV (trans → cis) or blue light (cis → trans) as well as by thermal relaxation (cis → trans).

We are developing the ‘molecular gate’ strategy based on the fact that most type II restriction endonucleases (RE) are homodimers [2]. The DNA-binding center is located in the interface between the two subunits. It is possible to modify the protein at the entrance of the DNA-binding site and block its activity. To create the obstacle for DNA penetration to the active center we suggest to use the ability of oligonucleotides containing azobenzene insertion to form a duplex. Azobenzene in trans-configuration stabilizes the duplex and cis-configuration causes destabilization [3]. Thus formation and dissociation of the duplex can be reversibly photo-regulated. The strategy is illustrated for the type II RE SsoII.

To choose the optimal length of the duplex 10-mer and 15-mer modified oligonucleotides were synthesized. After attachment to the protein these oligonucleotides are supposed to form 10-mer DNA duplexes. The initial rates of DNA cleavage at 37°C upon UV-illumination was two times higher than upon blue light illumination. Our results demonstrate the possibility of changing the enzymatic activity upon illumination.

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References


SW06.W33–24

DR5-B – DR5-selective mutant variant of cytokine TRAIL overcomes resistance of cancer cells to TRAIL

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Cytokine TRAIL (Apo2L) induces apoptosis by binding to death receptors DR4 and DR5 in broad spectrum of cancer cells while sparing normal cells, so recombinant TRAIL preparations are considered as potential anti-cancer drugs. However, many tumor cell lines are resistant to TRAIL, despite of DR4 and DR5 expression, partially due to the competition between death and decoy receptors (membrane-bound DcR1, DcR2 and soluble OPG) for binding to TRAIL. Recently, unique DR5-selective mutant variant of TRAIL (DR5-B) was generated in our laboratory. DR5-B practically doesn’t interact with decoy receptors and DR4. Here we show that DR5-B induces apoptosis 4-fold more effectively than wild type TRAIL in T leukemia Jurkat and monoblastic leukemia U937 cells expressing mainly DR5 receptor. DR5-B was also 1.3–10-fold more efficient in induction of apoptosis in TRAIL-sensitive human colorectal carcinoma HCT116 cells and TRAIL-resistant lung carcinoma A549, colon adenocarcinoma HT29 and breast adenocarcinoma MCF-7 cell lines which express all TRAIL membrane receptors. Combinational treatment of cancer cell lines with chemotherapeutic agents such as doxorubicin, paclitaxel and bortezomib (1–100 nM) and TRAIL or DR5-B preparations resulted in more than 90% apoptosis of Jurkat, HCT116 and U937 cells. Chemotherapeutic agents enhanced DR5-induced apoptosis of TRAIL-resistant cell lines A549, HT29 and MCF-7 much more than wild type TRAIL-induced cell death (maximal cell death 65–98% and 35–60% for DR5-B and wild type TRAIL respectively). Moreover in combinational treatment experiments effective concentrations of DR5-B were lower in all tested cancer cell lines in comparison to wild type TRAIL. Both preparations of DR5-B and wild type TRAIL were nontoxic to normal fibroblasts and endothelial cells (HUVEC) expressing all TRAIL receptors. These results demonstrate that DR5-selective TRAIL mutant variant DR5-B can be used for therapy of TRAIL-resistant cancers.

SW06.W33–25

Characterization of a lef8 knock-out BmNPV: new data for an old gene

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BmNPV is an insect infecting closed-circular dsDNA virus with a host spectrum restricted to the silkworm, Bombyx mori. As a model member of the Nucleopolyhedrosis Virus (NPV) family, it exhibits the typical 3-phase gene expression profile with early, late and very late transcripts and a virus-encoded multi-subunit RNA polymerase, required for the transition from the early to later gene expression. The lef8 gene, which encodes a subunit of this polymerase, is the target of this study: using the virus in bacmid form, we replaced the lef8 ORF with a zeocin resistance/YFP-reporter element by homologous recombination, thus creating a Δlef8 knock-out BmNPV. Previous data from a temperature-sensitive mutant of lef8 had suggested that the pathogenic capacity of the virus is disrupted at the restrictive temperature, while its replication competence is retained at levels similar to wild-type (wt). We now show that when the entire ORF is removed from the viral genome, the viral replication
capacity is also abolished, leaving the \(\Delta lef8\) bacmid as a persisting entity in \textit{Bombyx} Bn5 cell cultures up to the 160 h post-transfection. Rescue of the wt prototype by adenovirus of \textit{lef8} through extra-viral \(\Delta lef8\) gene-constructs (episomal or cell genome-integrated) or ectopic production from within the viral genome was not possible. Only restoration of the ORF (native or N-myc tagged either under heterologous promoter control or under the control of its native promoter sequences) in its original locus was able to effectively rescue the virus. These findings suggest a previously unidentified position-related function of the \(\Delta lef8\) ORF.

**SW06.W33–26**

**New insights into sugar catabolism of \textit{Arthrobacter nicotinovorans}: oxidative degradation of xylose**

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Due to its high abundance, the D-xylose fraction of lignocellulosic materials provides a promising energy resource [1]. Although theoretically, D-xylose can be readily exploited as a resource for production of ethanol or other chemicals, in practice such applications are scarce due to the lack of suitable enzymes. Known mostly due to its ability to metabolize nicotine, the actinobacterium \textit{Arthrobacter nicotinovorans} is a strong candidate for finding D-xylose degrading enzymes as it occupies an ecological niche rich in lignocellulosic materials: the nicotine-contaminated soil. Here, the pAO1 megaplasmid [2] was shown to be related to the ability of \textit{A. nicotinovorans} cells to ferment D-xylose. Three \textit{pAO1} genes (orf32, orf39, orf40) were identified, cloned and the corresponding proteins purified and characterized. ORF40 was shown to be a homo-tetrameric NADP⁺/NAD⁺-xylose-dehydrogenase (ALDH) able to oxidize various aliphatic aldehydes and ORF32 encodes a constitutively expressed transcription factor. Based on analogies with other pentose-degradation pathways, a putative oxidative pathway similar to the Weimberg pathway [3] is postulated: D-xylose is taken up by an ABC-type transporter and oxidised by XDH to D-xylonate, which may be further dehydrated by a putative xylonate-dehydratase (ORF41). The formed 2-keto glutarate semialdehyde could be further oxidised by ALDH to 2-ketogluturic acid. The latter compound can then be integrated into the general metabolism of the cell.

**References**


**SW06.W33–27**

**Some properties and utilization of fluorescent chimeras of human small heat shock proteins: disturbance of native protein properties inside of the chimeric constructs**

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Small heat shock proteins (sHsp) HspB1, HspB5, HspB6 and HspB8 are ubiquitously expressed in human tissues, where they function as molecular chaperones preventing aggregation of misfolded or partially denatured proteins. sHsp also participate in regulation of different vital processes such as protein folding and degradation, cell proliferation, apoptosis, cytoskeleton regulation. Fluorescent chimeras are often used for investigation of location and functioning of different protein \textit{in vivo}, however up to now biochemical properties of sHsp fluorescent chimeras were not thoroughly analyzed. We investigated properties of chimeras consisting of one of the four human sHsp and the enhanced cyan or yellow fluorescent protein (FP) fused to the N- or C-ends of sHsp. The wild type HspB1 and HspB5 form large oligomers containing more than 20 subunits. Fluorescent chimeras of these proteins form homooligomers containing smaller number of subunits and number of subunits and stability of oligomers decreased in the order: HspB1 WT→HspB1-FP→FP-HspB1, HspB5 WT→HspB5-FP→FP-HspB5. Chimeras of HspB6 and HspB8 form dimers or small oligomers, as well as the wild type proteins. The chaperone-like activity of fusion proteins was different from that of wild type proteins.

Homo- and hetero-FRET was used for analyzing subunit exchange of different sHsp and rate of exchange was estimated. It is concluded that the fusion of fluorescent protein alters the structure and properties of sHsp and that this effect depend on location of fluorescent protein inside of the chimeras.

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**SW06.W33–28**

**Biodiesel production from hazelnut oil by means of transesterification reaction**

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Fossil fuels are produced from unsustainable resources and there are limited resources of these fuels. Therefore, investigation of alternative renewable fuels is necessary. Bio-diesel is a renewable fuel and it is produced mainly from vegetable oils. In this study, firstly, optimization of transesterification reactions from hazelnut oil was studied for biodiesel production. To find suitable oil: alcohol ratio, hazelnut oil and methanol were tested in 1:2, 1:3 1:4, 1:5, 1:6, 1:8 and 1:10 molar ratio, respectively. In addition, suitable base catalyst concentration was researched for transesterification of hazelnut oil. 0.5, 1, 1.5, 2, 3 and 5% of potassium hydroxide was used for reaction and yields were calculated according to last product as bio-diesel. Characterization of fatty acid methyl esters in hazelnut were made by gas chromatography. The yields in 1:2, 1:3 1:4, 1:6, 1:8 and 1:10 molar ratio were found to be 42%, 54%, 73%, 82%, 78 and 71% respectively, and the highest yield was obtained in 1:5 molar ratio as 87%. Also, transesterification reactions were examined with different concentrations of KOH (0.5, 1, 1.5, 2, 3 and 5%) as a base catalyst. The maximum yield was obtained with 2% KOH and the
higher concentration of KOH increased the saponification reactions. As a conclusion, the highest yield of transesterification reaction was obtained from 1:5 molar ratio of huzelnut and methanol with 2% of KOH with the chemical composition of methyl oleate (82.34%), and methyl linoleate (11.28%), methyl palmitate (5.71%) and methyl stearate (2.37%). These transesterification reaction conditions will be tested for bio-diesel production from agricultural wastes.

SW06.W33–29
Cloning and expression of carbon cycle relevant enzymes of Ralstonia eutropha H16
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Ralstonia eutropha is a Gram-negative, strictly respiratory facultative chemolithoautotrophic bacterium which can use hydrogen and carbon dioxide as sole sources of energy and carbon in the absence of organic substrates. It has attracted great interest for biotechnology for its ability to degrade a large list of chloroaromatic compounds and chemically related pollutants. Moreover the production of biodegradable polymer polyhydroxalkanoates on an industrial scale has already been applied [1]. R. eutropha serves as a model organism for the mechanisms involved in the control of autotrophic carbon dioxide fixation, hydrogen oxidation and denitrification.

In our project the main objective is the cloning of different enzymes, like carbonic anhydrases and carboxylases, which allow the organism to fixate carbon dioxide. Important enzymes for the carbon dioxide fixation under lithoautotrophic growth conditions are Rubisco as well as PEP-carboxylase and pyruvate-carboxylase. These enzymes are not able to directly use carbon dioxide as substrate. Carbonic anhydrases are responsible for the conversion of CO₂ to HCO₃⁻, which can be used by these carboxylases. Four different carbonic anhydrases as well as the PEP-carboxylase and the Pyruvate carboxylase of R. eutropha H16 were cloned and expressed in Escherichia coli BL21. The specific enzyme activities of the carbonic anhydrases were measured by Infrared Spectroscopy. The enzyme activities of the carboxilases were determined spectrophotometrically in an assay linked to the NADH-dependent reduction of oxaloacetic acid. The overall aim of the study is the overexpression of carbonic anhydrases and carboxylases to provide a basis for efficient carboxylation reactions performed by R. eutropha H16 under lithoautotrophic conditions.

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SW06.W33–30
Cloning and characterization of a new dye degrading laccase from Bacillus amyloliquefaciens 12B1
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Industrial dyeing of textile consumes large amounts of water and energy, while 5-40% of the dyestuffs used are released in the effluent. Some dyes are recalcitrant to direct microbial degradation and there is an ongoing search for the introduction of clea-ner and more efficient technologies that will enable degradation of these compounds [1]. Promising new technology is the use of oxidative enzymes [2]. Laccases (EC1.10.3.2), a family of blue multicopper oxidasenes, are capable of oxidizing a wide range of aromatic compounds, with concomitant reduction of molecular oxygen to water [3]. Identifying and characterizing new laccases from bacteria and evaluating their application potential will greatly help us to better use them in industrial processes.

To obtain new laccases with ideal characteristics, one hundred soil isolated Bacillus sp. across Serbia have been screened for spore laccase activity. Ten percent of screened strains demonstrated laccase activity on their spores. A new laccase gene (cotA) was cloned from newly isolated Bacillus amyloliquefaciens strain and expressed in Escherichia coli. Temperature optimum, pH optimum and temperature stability were determined for both wt spore-bound laccase and recombinant laccase. The recombinant protein CotA demonstrated activity towards canonical laccase substrates ABTS, syringaldazine and 2,6-dimethoxyphenol. Highest oxidizing activity towards ABTS was obtained at 80°C. pH optimum of recombinant laccase is 5.0. Higher thermostability at 80°C was observed for recombinant enzyme. Oxidation of azo and aminochlorotriazine dyes was demonstrated and thus potential for industrial application confirmed. Due to high temperature optimum of this enzyme it is expected to use it in the treatment of hot effluents from textile industry.

References

SW06.W33–31
Escherichia coli F₀F₁-ATPase activity under glycerol fermentation at different pH and role of hydrogenases
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Escherichia coli is able to ferment glycerol and produce molecular hydrogen (H₂) by using different hydrogenases (Hyd) [1]. The important aspect in regulation of H₂ production by E. coli formate hydrogen lyase (FHL) and its energetics is the requirement of the F₀F₁-ATPase, which has been shown to be N,N'-dicyclohexylcarbodiimide (DCCD) sensitive during glycerol fermentation [2].

In this study overall and DCCD-sensitive ATPase activity of membrane vesicles was investigated with glycerol-fermented E. coli wild type BW25113 and mutant DHP-F2 (MC4100 ΔhypF) (with deficiency of Hyd-1; 2; 3 and 4) at different pH. ATPase activity of wild type strain was ~3-fold higher (p ≤ 0.05) at pH 7.5 compared with that at pH 6.5. Membrane vesicles ATPase activity was higher in wild type glucose-fermented cells ~1.5-fold at pH 7.5 compared with that in glycerol-fermented cells. DCCD inhibited markedly ATPase activity ~11-fold (p ≤ 0.05) at pH 7.5 and at pH 6.5 ~ ~1.3-fold (p ≤ 0.025). Compared with wild type cells, ATPase activity at pH 7.5 was decreased in ~2.2-fold (p ≤ 0.02) with ΔhypF mutant. DCCD inhibited ATPase activity of ΔhypF was lowered ~1.4-fold (p ≤ 0.025) at pH 7.5 and but not at pH 6.5.
The results obtained indicate that in \textit{E. coli} during glycerol fermentation alkaline pH is more optimal for the F$_{0}$F$_{1}$-ATPase activity. F$_{0}$F$_{1}$ has major input in overall ATPase activity. The suppression of ATPase activity in the \textit{hypF} mutant at pH 7.5 might be explained by some interaction between different components of Hyd with the F$_{0}$F$_{1}$-ATPase.

References

\textbf{SW06.W33–32}

\textbf{Diphenylene iodonium, as hydrogenase inhibitor, enhanced H$_2$ photoproduction by \textit{Rhodobacter sphaeroides}}

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Photosynthetic bacteria are often presented as perspective organisms for the molecular hydrogen (H$_2$) production, which might be mediated by nitrogenase, whereas hydrogenases may be active for H$_2$ uptake. In a previous report, we demonstrated the inhibitory effect of comparably high concentrations of diphenylene iodonium (Ph$_2$I), an inhibitor of hydrogenase, on H$_2$ production of \textit{Rhodobacter sphaeroides} [1].

In this study the effect of lower concentrations of Ph$_2$I on growth properties and H$_2$ photoproduction by purple non-sulfur bacteria \textit{R. sphaeroides} isolated from mineral springs in Armenia was investigated. This study can be helpful for identifying the responsible enzymes, understanding of mechanisms and pathways of H$_2$ production.

In this study Ph$_2$I has been shown to affect bacterial growth in a concentration-dependent manner by decreasing specific growth rate. Photosynthetic pigments have been found to be sensitive to the content of the growth medium. Absorption peaks typical for carotenoids and bacteriochlorophyll \textit{a} gradually disappeared in the presence of 1–5 mM Ph$_2$I. Redox potential of \textit{R. sphaeroides} measured by platinum electrode gradually decreased (up to $-710$ mV) during the growth at the presence of Ph$_2$I, which indicates H$_2$ production. The results point out the concentration dependent Ph$_2$I effect: 0.1–1 mM Ph$_2$I stimulated (-1.3 fold) yield of H$_2$ by \textit{R. sphaeroides}. H$_2$ production was not affected at the concentrations of Ph$_2$I up to 5 mM, and the inhibition of H$_2$ producing ability was enhanced with increase of Ph$_2$I concentration.

This result demonstrated that using the specific inhibitors of hydrogenase enhances H$_2$ production.

Reference

\textbf{SW06.W33–34}

\textbf{In vitro anticancer activity of levan from \textit{Halomonas snyrnessis} AAD6T}

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Levan is a bioactive fructan polymer that is mainly associated with high-value applications where exceptionally high purity requirements call for well-defined cultivation conditions (Kang et al., 2009). Halophilic \textit{Halomonas snyrnessis} strain was reported as the first extremophiliic producer of levan by our research group (Poli et al., 2009). Further research on the potential use of this fructan type polysaccharide as a bioflocculating agent (Sam et al., 2011), its uniform, homogeneous, nanostructured, biocompatible, thin films (Sim et al., 2012), its suitability for peptide and protein based drug nanocarrier systems (Sezer et al., 2011) and an economical production scheme via cheap carbon resources like starch and sugar beet molasses (Kucukasik et al., 2011) and sugar beet pulp were reported. In addition, the antioxidant potential of this levan polysaccharide in high glucose condition in the pancreatic INS-1E cells by demonstrating a correlation between reduction in oxidative stress and apoptosis with its treatment was reported for the first time by our research group (Kazak et al., in press).
sidering the ever increasing interest in levan and its potential applications in medical industry, the main aim of this study is to investigate the anticancer activity of this polysaccharide and its oxidized derivatives with different oxidation degrees in many cancer cell lines; such as MCF-7 (human breast adenocarcinoma), HepG2 (human liver hepatocellular carcinoma), A549 (human lung adenocarcinoma), AGS (human gastric adenocarcinoma), CaCo-2 (human colon adenocarcinoma). The WST-1 assay was performed for evaluating anticancer activity. As a result, oxidized form of levan with the highest oxidation degree showed the highest anticancer activity in all cell lines. The results suggested that levan derivatives could be explored as promising anticancer agents. These studies hold great importance for the medicinal applications of levan and its chemical modifications. Financial support: TUBITAK, Project No. MAG-111M232.

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SW06.W33–35

In vivo cell tracking by using bioluminescence imaging

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Bioluminescence imaging based on luciferin-luciferase reaction has been used as a powerful tool for in vivo cell-tracking studies. One of the most crucial issues is how to enhance the sensitivity of this method. The purpose of this work was to establish cancer and stem cell lines stably expressing enhanced firefly luciferase (luc2) and to track metastasis and distribution of stem cells in mice using bioluminescence imaging in vivo. We created the lentiviral vector containing luc2 and transfected the cells. The model of lung metastases was generated by intravenous injection of Colo 26-luc2 cells to BALB/c mice. Distribution of human stem cells labeled with luc2 was studied after their systemic administration to nude mice. The results showed that early metastases and small populations of stem cells can be detected by whole-body luc2-based bioluminescence imaging.

SW06.W33–36

Formate and growth medium composition influence on Escherichia coli growth and molecular hydrogen production under glycerol fermentation at different pHs

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Bioglycerol (H2) is known as an ecologically-clean, renewable and abundant alternative energy source for the further economy. Glycerol can be used by Escherichia coli as a carbon source for obtaining various valuable end-products including H2. The latter is being produced from formate, the end product of glycerol fermentation, probably by formate hydrogen lyase [3]. Influence formate in different concentrations of 10–50 mM on E. coli BW25113 growth and H2 production was investigated at different pHs (5.5–7.5) under glycerol fermentation. Ten molar formate 2-fold and 30 and 50 mM formate completely inhibited bacterial growth during glycerol fermentation at pH 5.5, whereas at pH 7.5 formate in the concentrations stimulated bacterial growth. H2 production kinetics during bacterial growth was different: in case of glycerol H2 was evolved at the middle log phase at 6.5 and 7.5 pHs while during glycerol and formate fermentation H2 production was observed and simulated 1.2 fold during early log phase at pH 7.5.

Bacterial growth on glycerol was also followed in minimal salt medium with inoculate grown on glucose and peptone: lag and log growth phases were prolonged at different pHs with 2-fold inhibition of bacterial growth. H2 production was determined starting 24 h growth and lasted until 72 h at pHs used; maximal yield and H2 production were observed at pH 7.5. It is worth to be mentioned that when bacterial inoculate did not contain peptone, H2 production was not observed in minimal salt medium. The findings point out the importance of medium composition for glycerol fermentation and H2 production by E. coli.

References

SW06.W33–37

Production of recombinant human growth hormone in Pichia pastoris

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Some of the cures of human growth hormone deficiency in children, Turner’s syndrome and AIDS associated catabolism are based on application of human growth hormone, the hormone also known as somatotropin. It is a multifunctional protein produced in a human brain by hypophysis. Somatotropin is known for its effects on metabolism and its promotion of growth of skeletal and soft tissues. The study tested approaches of increasing the yields of recombinant somatotropin and the effectiveness of its production by rational design of the expression system. The gene for somatotropin was synthesized according to the matured Isoform I of somatotropin containing 191 amino acids. This sequence had been chosen as canonical. The gene was inserted to the commercial expression vector pPICZaX designed for Pichia pastoris. The vector is determined to production of a recombinant protein to the media. It is caused due to the secretion signal sequence from the Saccharomyces cerevisiae factor prepro peptide. The cells produce very low levels of native proteins to the media. A combination with a minimal media makes purification much easier.

Pichia pastoris is well-suited for fermentative growth. A fermentation process is the way how to reach the high density of cells producing a recombinant protein which may improve overall protein yields.
This work is the result of the project implementation: ‘Production of biologically active agents based on recombinant proteins’ (ITMS 26240220048) supported by the Research and Development Operational Program funded by the ERDF.

**SW06.W33–38**

**Histidinyl phosphatidylethanolamine as an effective coplioid for transgene expression mediated by liposomal vectors**

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Liposomal transfection is a commonly employed approach of gene delivery for therapeutic purposes. Nevertheless, low levels of in vitro transgene expression limit its wide applications. In this study, two different acyl chains, distearyl and dioleoyl, of phosphatidyl ethanolamine were used to prepare histidinyl phosphatidyl ethanolamine, his-DSPE and his-DOPC, by grafting histidine onto the amine group of phosphatidyl ethanolamine. 1,2-diolen-3-trimethylammonium- propane (DOTAP) was used as the primary cationic lipid in the liposomes that contain DOPE, DSPE, his-DOPE, and his-DSPE as the colipid, respectively. Using his-DSPE could enhance the in vitro efficiency of green fluorescent protein expression with significantly reduced cytotoxicity when compared with DOTAP alone. When the vectors were administered to mouse by intravenous injection, both his-DOPE and his-DSPE showed increases in the luciferase expression of all the assayed organs including lung, heart, spleen, kidney, and liver. Among them, lung and spleen specifically showed most improved transgene expression. The levels of luciferase in the lung were enhanced by 77, and 54 fold for his-DSPE and his-DOPE, respectively, and those in the spleen were enhanced by 20 and 57 fold for his-DSPE and his-DOPE, respectively.

**SW06.W33–39**

**The new expression system based on a novel yeast species of the genus Komagataella**

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Species assignment of biotechnologically important strain VKPM Y-727 has been determined. Based on multigene sequence analyses (D1/D2 LSU rRNA, ITS1-5.8S-ITS2, mitochondrial small subunit rRNA, RNA polymerase subunit 1, translation elongation factor-1a) and phenotypic physiological properties, the strain was shown to represent a novel sibling species of the Komagataella (Pichia) pastoris. Phylogenetic analysis revealed that the new species is closely related to Komagataella phaffii. The strain VKPM Y-727 was shown to provide several technological benefits. Under conditions of methanol induction, its AOX1 promoter specific activity is 20–25% higher than the activity of the commonly used AOX1 promoter of K. phaffii. In VKPM Y-727, the derepressed AOX1 promoter activity is up to 70% of its methanol-induced level that makes possible promoter induction without using toxic and flammable methanol. Among other features is the methanol utilization phenotype of VKPM Y-727 that is intermediate between the well-known mut1 and mut2 phenotypes of K. phaffii. Besides, VKPM Y-727 and K. phaffii demonstrate somewhat different patterns of endogenous secreted proteins.

A series of auxotrophic mutants were derived from the strain VKPM Y-727, and expression vectors were constructed, in which inducible (AOX1) or constitutive (GAP) promoter drives expression of a target gene. Using this system a number of recombinant proteins were efficiently expressed including secreted hVEGF (biological active dimers) and HSA (>2 mg/ml, non-optimized fermentation) as well as intracellular HBsAg (4 mg/OD) and capsid L1 protein of HPV (serotypes 6, 11, 16, and 18) in amount of 12–20% of total soluble cell protein.

**SW06.W33–40**

**Development of transgenic Arabidopsis thaliana plants expressing a root-specific phytase of microbial origin**

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One of the major limiting factors of crop yield is insufficient quantity of inorganic phosphorus in the soil. To increase the productivity of plants, modern agricultural practices rely on rock phosphate fertilizer, but its use is inefficient, as ~90% of mineral phosphate is quickly converted to organic forms in the soil making it inaccessible to plants. A promising alternative direction in agriculture is utilization of abundant soil organic phosphorus compounds, such as phytate. Using secreted phytase enzymes, phytates can be easily broken down to phosphoric acid and myoinositol. While most plants do not possess secreted phytases, they can be engineered to secrete phytases of microbial origin. Thus, genetically modified plants that carry the genes for secreted bacterial phytases can be engineered to improve growth in phosphate-rich soils and offer a promising route in plant biotechnology.

The aim of this work was to obtain genetically modified A. thaliana plants carrying a genomic insert with the phytase gene of B. ginsengii (phyCg). The nucleotide sequence of the bacterial phyCg gene was previously optimized using CodonAdaptationTool (http://www.jcat.de/) to increase phytase expression in transgenic A. thaliana. Optimized phytase gene was cloned into the binary vector pCBK05 under the control of Pht1;2 promoter. Pht1;2 promoter is specific for the root epithelial cells of A. thaliana and is induced by inorganic phosphorus starvation. The resulting genetic construct also contained sequences encoding the signal peptide of the protein extensin (AtExt3) and the 3’-terminal His and Strep sequences. Transformation of A. thaliana was carried out by dipping the flowers of A. thaliana into a suspension of Agrobacterium tumefaciens GV3101 cells. Selection of transformants was carried out on MS medium supplemented with selective herbicide BASTA and identified 28 primary transformants, from which second generation plants were also obtained. The presence of transgene insertion in the plant genome was further confirmed by PCR. Further analysis of obtained transformants will help design an improved transgenic approach for the development of a root-specific heterologous system for the expression of bacterial phytases in plants, facilitating the transition to a more sustainable agriculture.

The work is supported by the Federal target program ‘Scientific, research and educational personnel of innovative Russia’ № 14.A18.21.0849.
SW06.W33–41
Plant growth-promoting fungi from soils under different agricultural crops
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Potential object of agrobiotechnology is PGPF (Plant Growth-Promoting Fungi) which are frequently used as inoculants for improving the growth and yield of agricultural crops. The positive influence of soil fungi on plants is also related to their ability to suppress pathogenic microorganisms, which could inhibit plant growth. The application of PGPF is an attractive alternative to chemical fertilizers.

Objective of the present study is selection of the most active fungal strains with ability to promote plant growth and inhibit phytopathogenic fungi.

Studies were conducted with 188 strains of fungi isolated from soils under different agricultural crops – alfalfa (Medicago sativa), melilot (Melilotus officinalis), saffoin (Onobrychis vicifolia), and rapeseed (Brassica napus). To determine plant growth promoting ability the alfalfa seeds were treated with culture filtrates of fungal isolates. The seedling emergence, shoot and root length of treated plant seedlings were compared to nontreated ones. The antagonistic activity of strains against phytopathogenic fungi Fusarium graminearum and Alternaria alternata was studied by dual culture method.

Based on the phenotypic and phylogenetic characteristics it was shown that all fungal isolates belonged to 19 species of five genera – Penicillium, Fusarium, Aspergillus, Trichoderma and Acromonim. Thirty-four strains had the plant growth-promoting ability. Seedling emergence reached 99.6%. The root length increased by 27.4–65.2% compared to control plants. The shoot length was higher by 19.4–64.7% compared to the control. Results of the antagonism tests showed that 21 isolates demonstrated the ability to inhibit the growth of phytopathogenic fungi. The size of the inhibition zone was 1.5–9.5 mm. It was found that the antagonistic activity was higher against Fusarium graminearum. The largest number of active strains was typical for soil under alfalfa. Eleven of the most active strains of species Lus ustus were selected for further study.

SW06.W33–42
Cytocompatibility of Ar plasma treated polyhydroxybutyrate for fibroblasts and keratinocytes: adhesion molecules in action
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The cell-material interface plays a crucial role in the interaction of cells with synthetic materials for biomedical use. The application of plasma for tailoring polymer surfaces is of abiding interest and holds a great promise in biomedicine. We studied the surface properties of pristine and Ar plasma treated polyhydroxybutyrate (PHB) by different methods and it’s cytocompatibility as a function of the plasma discharge power and period of the plasma exposure. The Ar plasma treatment of PHB resulted in changed polymer surface polarity, roughness and morphology, which we thoroughly characterized (by atomic force microscopy, goniometry and photoelectron spectroscopy). The effect of our modifications on adhesion and growth was tested in vitro using mouse embryonic fibroblasts (NIH 3T3) and human keratinocytes (HaCaT). We demonstrate that the plasma treatment of PE had a positive effect on the adhesion, spreading, homogeneity of distribution and proliferation activity of NIH 3T3 and HaCaT cells. By immunochemical and immunofluorescent methods, we have also determined the response of adhesion molecules talin and vinculin on the type of PHB treatment.

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SW06.W33–43
Bioengineering of bacteria for removal of haloacids
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Halocids are environmental pollutant and can be generated during disinfection of water or as metabolic products of microbial transformation of complex halogenated organic compounds. Bacteria capable of utilizing haloacid can be found in the natural environment. These bacteria possess enzyme known as dehalogenase that removes the halogen hydrolytically and renders the products suitable for general metabolism. A soil bacterium, Burkholderia caribensis strain MBA4, was isolated and found to produce an inducible dehalogenase. When the dehalogenase gene deh4a and corresponding upstream noncoding region, was cloned and transformed to related species such as B. phymatum it allowed the host to utilize haloacid as a growth substrate. This recombinant was shown to produce 5-times the amount of dehalogenase in the wildtype. While this result seems encouraging, the specific activity was far from exceptional. In this presentation, we would like to describe the production of this dehalogenase in the model organism Escherichia coli, with an aim to evaluate the bioremediation potential of bacteria. The dehalogenase gene was amplified from the genome of MBA4 and cloned into two inducible expression vectors – viz pRSET and pPRO-EX-HT. Cell extracts were prepared from cells grown in medium with or without inducer and the specific dehalogenase activities determined.

SW06.W33–44
Improved chondrogenic capacity of collagen hydrogel-expanded chondrocytes: in vitro and in vivo analysis
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Background: The use of autologous chondrocytes in the repair of cartilage is limited due to the losing of cartilage phenotype during expansion. Their mechanosensing capacity prompts to the use of soft substrates for the in vitro expansion. Our aim was to test the expansion of chondrocytes using collagen hydrogels in order to improve their capacity for chondrogenesis after a number of passages.

Methods: We expanded rat cartilage cells using collagen hydrogels and test the preservation of their chondrogenic capacity both in vitro and in vivo. We measured the expression of markers Type II collagen (Col2), Sox9, FGFR3, Alk5, Alk1 and BMP2 during the expansion on both surfaces by RT-PCR.
**Response of cartilage cells to changes in the mechanical environment in vivo**

**Background:** Response of cartilage cells to changes in the mechanical environment is essential to understand the physiology and repairing capacities for chondrocytes. It has been recently described but little is known about the underlying mechanisms. The aim of this work was to determine the pathways implicated in the mechanosensing of chondrocytes.

**Methods:** Rat chondrocytes were cultured in collagen hydrogels of different stiffness (2–20 Pa) in normoxia and hypoxia, in monolayer and embedded inside hydrogels. The participation of integrins was determined by integrin blockade experiments as the first event in the mechanosensing. Expression of proteins member of the Focal Adhesion complex was also analysed in our model, as well as phosphorylations and potential interactions as it will be indicated in the results section. RT-PCR, western blot and immunoprecipitation were used for these experiments. Finally, as suggested by the results of gene expression during expansion of cells in soft hydrogels, the role of the receptors Alk-1 and Alk-5 was also related with the effect of substrate stiffness on chondrocytes.

**Results:** We identified Non Muscle Myosin II (NMMII) and integrins a1, b1 and b3 as participants in the mechanosensing, since their blockade inhibits the sensing of the stiffness. RT-PCR arrays and western blot detected up-regulation of Paxillin, RhoA, Fos, Jun and Sox9. Src seemed to have a role in the 2D/3D switch in vitro, while FAK showed variations in its behaviour in normoxia or hypoxia. These variations could have been due to interaction with HIF-1, as it is suggested by immunoprecipitation experiments. Alk-1 and Alk-5 is strongly related with the final phenotype of the cell. Overexpression and silencing experiments allowed us to demonstrate that the ratio for these receptors is modified during soft substrate culture and is one of the responsible mechanisms for the final effect of the mechanical component.

**Conclusions:** We determined here some of the genes that are up-regulated during the process of chondrocyte mechanosensing.

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**SW06.W33–46 Interference from a myeloma derived light chain on diagnostic monoclonal antibody performance**

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Hybridoma-derived antibodies are used widely in the development of diagnostic assays. In the past, myeloma fusion partners expressing endogenous heavy and/or light chains were often used to generate hybridoma cell lines. As a result, mixed populations of antibodies would often be purified and cause inaccurate results when tested. In this report, we describe one such scenario where a P3U1 myeloma fusion partner was used in the generation of a hybridoma that secreted three subpopulations of immunoglobulins as assessed by ion exchange chromatography and mass spectrometry. The subpopulations also exhibited varying degrees of immunoreactivity (0, 50, or 100%) to the target antigen as determined by surface plasmon resonance and an automated chemiluminescent-based immunoassay. To produce an antibody with the highest possible specific reactivity, the variable domains of the heavy and light chains of the antigen-specific antibody were cloned onto mouse IgG1, IgG2a and human IgG1 Fe-region scaffolds and expressed recombinantly in Chinese hamster ovary (CHO) cells. The purified recombinant antibodies demonstrated far superior performance compared to the IgG1 antibody produced by the parent hybridoma.

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**Engineering novel crystallization chaperones with tunable crystal packing and increased power of phasing**

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DARPin [1,2] are widely used as crystallization aides [3,4] and well known for their stability, high expression yield, and their ability to crystallize under various conditions. Moreover, DARPins can be used as a search model in molecular replacement to address the phase problem. Despite all advantages, DARPins sometimes fail to provide the necessary polar surface to form crystal contacts due to their small size in comparison to the target protein.

To extend the range of potential applications of the DARPin technology, we have developed a new class of molecules: DARPin-beta-Lactamase fusions. In this design, the C-terminal helix of the C-capping repeat of a DARPin extends and continues as the N-terminal helix of its fusion partner, beta-Lactamase. The key feature of this design is that the fusion partners are both stable, well expressed proteins fused rigidly, via the well-defined shared helix and therefore behave as one domain. Fusing the DARPin in a rigid manner with larger, soluble proteins extends the possibility of crystal contact formation. In addition, there are not only one but six variants of the fusion protein, which vary in terms of relative orientation of the DARPin part to the beta-Lactamase part. This brings another level of control upon the crystal formation properties of newly designed scaffolds, and is part of an ongoing effort to build a toolbox for crystal formation.

In this work we present the engineered DARPin-beta-Lactamase fusion design and crystal structures of four members (1.4–2.1 Å), two of which are in complex with the target protein. The fusion framework is generic and can be used with any DARPin, and can therefore be adapted to any target protein.
The first step of the work was collecting B-polluted waters from strategic importance. B reserves of 60%, 800 million tonnes. Thus B removal is of strategic importance both in terms of being eco-friendly and low-cost. Turkey is the world's leading country in respect to human health through irrigation, drinking water and food chain. In this context, high levels of B in the soil have an impact on this context, high levels of B in the soil have an impact on high concentrations, but at high concentrations it is very toxic. In this context, high levels of B in the soil have an impact on this context, high levels of B in the soil have an impact on.

The determination of boron removal capacities of newly isolated microorganisms from boron-contaminated waters

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Boron plays an important role in many biological reactions at low concentrations, but at high concentrations it is very toxic. In this context, high levels of B in the soil have an impact on human health through irrigation, drinking water and food chain. In recent years, using of biological materials in B removal process is considerably important both in terms of being eco-friendly and low-cost. Turkey is the world’s leading country in respect to B reserves of 60%, 800 million tonnes. Thus B removal is of strategic importance.

In the present study we aim at the determination of the highest B removal capacity microorganisms through various parameters. The first step of the work was collecting B-polluted waters from Eti Mining Operations General Directorate, Turkey. Water samples were transferred to Petri plates which contain MSM-agar (minimal salt medium) and certain B concentrations. Pure isolates were obtained through continuous passaging of microorganisms. 18S rRNA gene amplification analysis revealed that one of our isolates is Rhodotorulla mucilaginosa and the other is Penicillium crustosum. At the second step of the work, isolates were cultivated in 1 ml liquid MSM media at a pH range of 4–7 for a week in an orbital shaker at an agitation rate of 100 rpm and incubation temperature of 30 ± 1°C.

The effects of pH concentrations on B removal were investigated as 19.76, 10.23, 13.57 and 10.05% for R. mucilaginosa and 19.97, 10.57, 11.37 and 11.75% for P. crustosum at pH 4, 5, 6 and 7, respectively. The maximum B removal yield was achieved at pH 4 for both of the microorganisms. The last step of the study is the determination of the relation between B removal and increasing B concentrations and also different culture media compositions. At the end of the work we want to reveal that the isolated microorganisms might be effective biomaterials on boron removal process.

Keywords: Boron bioremoval, Rhodotorulla mucilaginosa, Penicillium crustosum, boron-polluted waters.

Genetically encoded fluorescent indicator for NAD+/NADH ratio imaging in different cellular compartments

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The ratio of NAD+/NADH is a key indicator that reflects the overall cellular redox state. Until recently, UV and two-photon microscopy were the only methods for NADH imaging of the living cells in real-time. However these methods do not discriminate NADH from oxidized flavins and fail to report NAD+/NADH pair redox state. Recently, genetically encoded NAD+ sensors were reported. However, their sensitivity ranges are not optimal for mitochondrial NAD+/NADH detection. We developed RexYFP, a genetically encoded probe for NAD redox state, by inserting circularly permuted YFP into redox sensor T-REX from Termus aquaticus. RexYFP has several advantages over existing sensors. It has a smaller size and works in both the cytoplasm and the mitochondria.

Fluorescence of RexYFP depends on pH as in other sensors based on cpYFP. However, we developed an efficient strategy of eliminating pH-associated changes of the RexYFP signal. Using this strategy we detected changes in cytoplasmic NAD+/NADH associated with pyruvate and lactate changes in the medium on the background of acidification of the cellular cytoplasm. Mitochondria are the major NADH consumers in the cell. RexYFP detected a rotenon-induced increase in cytosolic NADH indicating that reduction of NAD fraction in the matrix spreads to the cytoplasm. Inhibition of the respiratory chain led to slower NAD pool reduction compared to lactate addition. Addition of an excess of H2O2 to the same cells after incubation with rotenone led to the rapid oxidation of NAD pool. Complex II inhibitor 3-nitropropionic acid caused decrease of NADH in the mitochondrial matrix while NAD+/NADH ratio in the cytoplasm remained stable. The mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazon (CCCP) led to oxidation of NADH. This effect was more pronounced in the mitochondria than in the cytoplasm. Subsequent addition of 25 µM rotenone to the same cells caused the increase in NADH in both compartments. Nota-
bly, the effect of the rotenone was stronger in the cytoplasm than in mitochondria. Taken together, RexYFP is a valuable tool for NAD$^+$/NADH imaging in different compartments of the living cell.

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SW06.W33–51
Biotherapeutics with improved pharmacokinetic properties
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Multiple technological approaches have been applied over the last decades in order to improve the pharmacokinetic (PK) profile of therapeutic proteins and peptides: i) conjugation of polyethylene glycol chains (PEGylation) to increase the hydrodynamic volume of the molecule; ii) employing FeRn-mediated recycling by fusion with the IgG Fc-region or coupling with serum albumin. A number of therapeutic polymer-protein conjugates are produced with the use of PEGylation technology. However, this approach is often limited by side effects caused by PEG accumulation in kidney or other tissues, decreased protein activity and manufacturing hurdles which seriously raise the cost of PEG-coupled drugs.

Alternative technologies, like PEG-mimetics, have recently emerged to substitute PEGylation. For instance, the PASylation technology developed by XL-protein involves the genetic fusion of the therapeutic protein with an amino acid polymer comprising proline, alanine and serine. PAS sequences adopt random coil conformation with an expanded hydrodynamic volume, thus retarding renal filtration in a similar way as PEG. Plasma half-life of the PASylated proteins can increase up to 10–100 times depending on the length of the polymer. In addition, PAS sequences are resistant to serum proteases but can be rapidly degraded by kidney proteases. Hence, PASylation allows to develop biologics with similar pharmacological properties but less side effects compared to PEGylation.

Among other products IBC Generium is developing Interferon β-1b coupled with PAS sequences for treatment of multiple sclerosis (MS) in patients with relapsing-remitting MS. Despite the huge therapeutic potential there is a limitation that hinders its clinical use: short circulation half-life in plasma which necessitates frequent administration (e.g., s.c. injection every 2 days). Thereby, the development of a drug with prolonged action is in great demand. IBC Generium recently entered into a collaboration with XL-protein to validate several PASylated IFNβ versions in vitro. Up to now, we have successfully identified a candidate for further development. The antiproliferative activity of the molecule is similar to the reference drug. Currently, we are about to determine the PK properties in a relevant animal model.

SW06.W33–52
Efficient system of obtaining genetically modified primordial germ cells for oviduct expression in chickens
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Avian primordial germ cells (PGCs) have been isolated from blood (bPGCs) or gonads (gPGCs) of embryos at various stages of development, using different purification techniques which included: Ficoll, Nycodenz, Percoll density gradient centrifugation, ammonium chloride-potassium (ACK) lysis buffer, immuno-magnetic cell sorting (MACS) and fluorescence-activated cell sorting (FACS). Such PGCs can be transfected in vitro, based on the use of avian retroviruses or non-viral methods and injected into recipient embryos. We used non-viral ovalbumin promoter system to obtain chicken oviduct bioreactors (for human interferon alfa- hIFNγ gene and antigen HBsAg). We have developed successful method of isolation, purification and transfection of PGCs. The highest average percentage of transfected bPGCs (75.8% for EGFP) resulted in the centrifugation of the Percoll density gradient and electroporation. After ACK treatment and lipofection, the transgene EGFP expression in vitro was detected only in 35.2% of bPGCs. The chimeric chickens were produced from these cultured and modified cells, expressed in the gonads of the EGFP gene (it was detected in the gonads of 44% of recipient embryos injected with hPGCs). The generation G0 of birds was screened: blastodermal cells DNA (4.9% and 16.7%) and sperm DNA (3.5% and 2.4%) for the transgenes: hIFNγ gene and antigen HBsAg using a PCR assay, respectively. The identified transgenic chickens were used for generating G1 and G2. In the next step, we will collect eggs laid by transgenic G1 and G2 hens to test them for the presence of recombinant protein in egg white assay by ELISA.

SW06.W33–53
Genetically encoded red fluorescent probe for intracellular H$_2$O$_2$ detection
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HyPer is a modern genetically encoded fluorescent probe for intracellular hydrogen peroxide detection. The main advantages of HyPer are high selectivity, reversibility, specificity, rapid response to hydrogen peroxide, subcellular targeting and transgenic expression options. However HyPer has green emission which overlaps with most of the currently existing fluorescent protein-based sensors. Expanding the spectral range of the HyPer probes to longer wavelengths would enable hydrogen peroxide detection in multiparameter and in vivo imaging.

We have developed the first genetically encoded red fluorescent sensor for hydrogen peroxide detection, HyPerRed. HyPer consists of regulatory domain of H$_2$O$_2$ sensing transcriptional factor of E. coli OxyR with inserted circulary permuted YFP. We replaced cpYFP in HyPer to cpmApple, a circularly permuted red fluorescent protein from a red fluorescent calcium sensor R-GECO1. By high-throughput screening of semimimized sensor libraries we selected a clone called HyPerRed that demonstrated 80%-increase of fluorescence in response to hydrogen peroxide. HyPerRed sensitivity to H$_2$O$_2$ is the same as of green HyPer.

Cys199 is a critical residue reacting with H$_2$O$_2$ and forming disulfide with Cys208 in both HyPer's. To proof that the same residue mediates HyPerRed response we substituted Cys199 to Ser. Having the same spectral properties as HyPerRed, HyPer-Red-C199S was not sensitive to H$_2$O$_2$, demonstrating that the changes in fluorescence in HyPerRed upon oxidation by H$_2$O$_2$ are mediated by the same redox reaction as in HyPer and OxyR.

We tested HyPerRed expressed in the cytoplasm of HeLa Kyoto cell in culture by adding exogenous hydrogen peroxide and by physiological stimulation with epidermal growth factor.
(EGF). In both experiments HyPerRed demonstrated responses similar to green Hyper variants. The sensor with C199S mutation demonstrated no response to EGF or external hydrogen peroxide addition in HeLa-Kyoto cells. Finally, we demonstrated utility of HyPerRed in multiparameter imaging of Ca$^{2+}$, H$_2$O$_2$ and EGFR internalization. Taken together, HyPerRed is a suitable sensor for H$_2$O$_2$ detection in prokaryotic and eukaryotic cells.

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SW06.W33–54
Investigation of Gonium sp. biomass in possible usage of removing Reactive Blue 220
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Several industries have produced many kinds of outcome. In this way, wastewaters containing many pollutants were generated because of this production. The effluents sourced from industries contain different kinds of pollutants ranged from heavy metals to several dye stuffs. These pollutants are highly toxic to the environment when they are not treated from contaminated areas. Of these, reactive chemicals such as reactive dyes are very toxic, mutagenic, and carcinogenic. In the wastewaters including such pollutants light transition is prevented by these reactive dyes and aquatic life is affected negatively. Reactive dyes also have a resistance to degradation according to their aromatic structure. This property of reactive dyes makes them very resistive to the natural degradation. It is because of these properties, wastewaters containing reactive dyes have to be treated. In the current study, Gonium sp. biomass was investigated if it had a capacity in removing Reactive Blue 220 (RB 220) in BG11 media. The alga used in this study was obtained from Ankara University, Faculty of Science Laboratories’ current culture collection. Reactive Blue 220 was obtained from C. tropicalis sp. biomass in terms of different pH levels (6–10), initial biomass concentration was increased, dye removal also increased. Reactive dye also have a resistance to degradation according to their aromatic structure. This property of reactive dyes makes them very resistive to the natural degradation. It is because of these properties, wastewaters containing reactive dyes have to be treated. In the current study, Gonium sp. biomass was investigated if it had a capacity in removing Reactive Blue 220 (RB 220) in BG11 media. The alga used in this study was obtained from Ankara University, Faculty of Science Laboratories’ current culture collection. Reactive Blue 220 was obtained from Çiçek Textile Industry and Trade Co. Ltd., Denizli, Turkey. The experiments were carried out with Gonium sp. biomass in terms of different pH levels (6–10), initial dye concentrations (25–100 mg/l), and initial biomass concentrations (0.21–0.53 g/l). According to the data obtained from the experiments, Gonium sp. removed the applied RB 220 (nearly 40 mg/l) with the highest yield as 54.2% at pH 8. Removal of the dye by Gonium sp. was very high at 26.2 mg/l dye concentration as 84.2% (pH 8). In trails, it was determined that when biomass concentration was increased, dye removal also increased. The maximum removal capacity was found nearly 97% with 0.53 g/l biomass under approximately 25 mg/l dye concentration.

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SW06.W33–55
Investigating transesterification reaction parameters of Candida tropicalis lipids for biodiesel production
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The biggest drawback for commercialization of biodiesel is the high cost of raw material, mainly due to vegetable oil. In this context microbial oils which are formed from long-chain fatty acids similar to plants oils, are comparable to oils are promising feedstocks for producing biodiesel since their cheapness and high level of lipid content.

In the present study various key parameters of reaction conditions that are affecting the biodiesel production capacity of a yeast (C. tropicalis) have been described. Molasses was used as a carbon and energy source in the experiments to reduce the cost of lipid production.

In order to examine the effect of catalyst type on the transesterification reaction, acidic (sulphuric acid), and basic (potassium hydroxide and sodium hydroxide) catalysts were used in the study. To see the effect of alcohol type on the transesterification reaction, methanol and ethanol were used in the presence of a catalyst. To investigate the amount of alcohol and catalyst on the reaction, increasing alcohol:oil (2.5–10.0) and catalyst:oil ratios (0.2–0.8) were added to the reaction mixture. To see the effect of reaction time courses on fatty acid methyl ester yield 2, 6, and 24 h of reaction time were tried. Fatty acid profiles of microbial oils were performed by gas chromatography after lipids were transesterified to biodiesel by an alcohol and a catalyst.

Higher fatty acid methyl ester yields were obtained when C. tropicalis lipids which transesterified with acidic catalyst and methanol. The highest fatty acid methyl ester yield was obtained as 99% in the presence of 10.0 methanol:oil, 0.8 sulphuric acid: oil at 30°C for 6 h. It was concluded that increase in the quality of a catalyst did not change the yield and the quality of the esters dramatically.

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SW06.W33–56
Using the biomass of halophilic Dunaliella sp. microalgae for bioethanol production
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In today’s world petroleum based fuels are decreasing day by day because of their rapid consume. Besides their limited amount, their usage causes some problems to the environment. In this context, the researchers have been focused on finding alternative energy sources. The biofuels that derived from renewable biological sources (for example biodiesel and bioethanol) are promising solutions to solve the increasing energy problems.

In the current study microagal biomass was used as a raw material for bioethanol production. Dunaliella sp. strain was used in the study, as provided by Ankara University, Faculty of Science Laboratories’ from the current culture collection. The microagal cells were cultured in 250 ml Erlenmeyer flasks containing 100 ml Johnsons fermentation medium. Different physical (sonication, temperature with pressure) and physicochemical methods (acid hydrolysis with and without autoclave) were tested to investigate the most appropriate pre-treatment procedure for microagal cells. Furthermore, different parameters such as nutrient limitations and pH values were examined for increasing the carbohydrate content of the cells.

Saccharomyces cerevisiae yeast was used for microagal fermentation. S. cerevisiae was obtained from Ankara University, Faculty of Science Laboratories’ from the current culture collection. The yeast cells were cultured in YPG medium at pH 5. One percent yeast suspension was aseptically transferred to the anaerobic fermentation medium which is prepared by microagal sugar containing distilled water.

The bioethanol concentration was analyzed using gas chromatography (Shimadzu, Model GC-14B). The sugar concentrations were determined by phenol-sulphuric acid method. The microalgal biomass was harvested at the end of fifteenth day of incubation time and pretreated with 1% H$_2$SO$_4$ and autoclaved, and 0.316 g/l bioethanol yield was observed.

Keywords: Bioethanol, microalgal, biomass, biofuel.
Bacterial proteins for specific binding of fluorogenic synthetic dyes

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Fluorescent dyes are widely used for visualization of structures and processes within live cells. Popular tools for live cell imaging are fluorescent proteins and site-specific chemical labeling systems. Development of new protein-dye pairs is promising, especially with dyes which are non-fluorescent otherwise than in complex with protein.

We selected several E. coli proteins which have cavity suitable for binding Kaede-like chromophores, for instance, enzymes of glycolysis and fatty acid synthesis. Selective reversible binding of chromophores with some proteins was observed in vitro. Notably, intensity of chromophore-protein complex’s fluorescence increased at least ten-fold in comparison to fluorescence of free chromophore.

Method for light-induced separation of protein domains via backbone cleavage

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Optogenetics is a recently introduced groundbreaking methodology to control cell functions (neural activity, signaling cascades, protein interactions, etc.) with light via genetically encoded light-sensitive proteins. Here we developed new optogenetic method for light-induced separation of protein domains from a single polypeptide chain. The method utilizes green-to-red photocleavable fluorescent protein Dendra2, which acquires protein backbone break after photoconversion. However, in spite of broken polypeptide chain, Dendra2 retains its native 3D structure due to multiple non-covalent interactions of the halves of barrel. To achieve separation of the protein halves, Dendra2-containing chimeric proteins were targeted to intracellular unfolding molecular machines such as proteosome and mitochondrial import complex. As a result, after Dendra2 photoconversion we achieved physical separation of the N- and C-terminal domains that was detected in live cells by confocal microscopy due to translocation of CFP-labeled N-terminal part from cytosol to nucleus. We believe that the new method opens opportunities of light-induced translocation of target proteins into nucleus to control promoter activity or recombination events.

Biotechnological production of recombinant analogues of natural thrombin inhibitors from different haematophagous animals

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Successful application of analogues of natural thrombin inhibitor hirudin-1 from the medicinal leech Hirudo medicinalis (desirudin, lepirudin, bivalirudin) for the treatment of thromboembolic disorders stimulated the search for new natural highly specific anticoagulants, development of their recombinant analogues and study of their antithrombotic potential.

The present work is devoted to the production and study of recombinant analogues of several natural direct inhibitors of thrombin: hirudin-1, haemadin from the landliving leech Haemadipsa sylvestris, anophelein from the mosquito Anopheles albimanus and variegin from the tropical bont tick Amblyomma variegatum. We used modified mini-inteins (DnaB from Synechocystis sp. for hirudin-1, and GyRA from Mycobacterium xenopi for the three other peptides) as N-terminal or C-terminal fusion partners for the target polypeptides. In the constructions with hirudin-1, anophelein and variegin intein was located at the N-terminus of the corresponding fusion proteins, and their self-cleavage was pH-dependent. Similar construction was not suitable for the isolation of variegin, since its first amino acid is Ser, which strongly promotes uncontrollable cleavage of the fusion protein in vivo. So intein MxeGyRA was fused to the C-terminus of variegin, and the cleavage of this hybrid protein was induced by a thiol reagent. A unified method for the production of all the three target proteins, including isolation and cleavage of the fusion protein and purification of the target product by anion exchange chromatography and RP HPLC, was developed.

Antithrombotic activities and inhibition constants of the obtained recombinant analogues were determined by an amidolytic assay based on the ability of thrombin to hydrolyze a specific chromogenic substrate. As expected, the recombinant haemadin (284 ± 4 IM and 17 616 ATU/mg) was nearly as potent as the recombinant hirudin-1 (240 ± 4 IM and 19 802 ATU/mg). Though variegin (340 ± 3 PM and 674 ATU/mg) and anophelein (87 ± 3 PM and 3363 ATU/mg) were less potent thrombin inhibitors, they may be considered as an alternative to bivalirudin, a synthetic analogue of hirudin-1 with a Ki of 2.9 nM.

Antibacterial properties of charged-stabilized silver nanoparticles

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The charge-stabilized silver nanoparticles were synthesized via the chemical reduction processes. The surface properties of the particles were changed using various reducing agents, both inorganic (sodium hypophosphite and sodium hexametaphosphate) and organic phenolic compounds (gallic acid – GA, tannin –TA). All silver sols were thoroughly characterized by various physico-chemical methods such as TEM, SEM, AFM, DLS and micro-electrophoresis. Toxicity effect of silver nanoparticles against Escherichia coli K12 and antibiotic resistant strain was evaluated in terms of the Minimum Bactericidal Concentration (MBC). A pronounced bactericidal effect against the standard K12 strain was found for the GA and TA sols characterized by MBC concentration as low as 1–5 mg/l. On the other hand, in the case of the tetracycline-resistant E. coli strain, the highest activity (MBC of 10 mg/l) was observed for the sol synthesized using sodium hypophosphite and sodium tripolyphosphate. These results strongly indicate that the surface chemistry of silver nanoparticles is an important factor in destroying bacteria cells.

The mechanism of biocidal silver nanoparticle activities and their interactions with cell membranes were also studied using TEM and AFM imaging. It was unequivocally shown that the nanoparticles attach to the bacteria membranes, inducing their disintegration, which enables their penetration inside the bacteria.
The biocatalytic activity of synthesized, charge-stabilized, surfac-
tant-free silver nanoparticle sols can be exploited for preparing
biomaterials and antibacterial coating such as: catheters, wound
dressing and various household products.

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**SW06.W33–61**

**Phytase of Bacillus sp. M2.11: cloning, expression and purification**

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Nowadays feed additives connect farm animals feeding with obtaining
with a high level of productivity and product quality. Phytase
is used as a feed additive for degradation of antinutritional phytate [Tran T.T., Mam G., Mattiasson B., Hatti-Kaul R. A
thermostable phytase from Bacillus sp. MD2: cloning, expression
and high-level production in Escherichia coli][3 Ind Microbiol
Biotechnol. 2010. V. 37. P. 279–287]. These enzymes don’t pro-
duce in digestive tracts of pigs, poultry and other monogastric animals. That’s why phytic phosphorus passes unchanged throw
the digestive track of the animals and comes with a litter which
applies in soil later. Microbial phytases split phytates and convert phosphorus and associated metal ions in condition available for
animals. Despite the fact that phytases of some microorganisms
are isolated and described the history of these phosphatases is still
far from the completion. The maximum suitable for use phytases
are not found. Therefore, the constant search of new phytate
s producers is still relevant. Phytases can be used in biotechnology
and food industry. Production of phytase on a commercial scale became possible only by using appropriate genetic modific-
aton of microorganisms.

The aim of the work was to purify recombinant phytase of
Bacillus sp. M2.11. Microorganism Bacillus sp. M2.11 showing phytase activity was isolated from soil samples of different agri-
cultural companies of Republic of Tatarstan. Further these
strains were identified by 16S rRNA sequence. Thereby it was
known that one strain of the bacteria belongs to Bacillus sp. For
identification and comparative analysis of the genes encoding phytase, bacterial genomes of Bacillus genus were analyzed by
data represented on the server of NCBI (National center for biotech-
nology information) database. Results of phyBg genes’ sequence alignment presented in the international databases
revealed a high degree of homology of these genes between bacil-
lary cells. We constructed two primer pairs for amplification. As
a result, we obtained PCR fragment of DNA. Size of phytase
gene corresponded to 1149 base pairs. During the optimization
of amplification conditions it was found that the optimum tem-
perature is 60°C. This temperature increases the specificity and
efficiency of annealing. For bacillary phytase production is used recombinant strain Escherichia coli resistant to chloramphenicol
and carbencillin. Strain obtained by transformation of pET-LIC-
Phy plasmid in the protease-deficient strain of Escherichia coli
Rosseta 2 (DE3) T1R (Novagen, Germany), which proteinases
genes were deleted from the chromosome. Multicopy plasmid
pET-LIC-Phy, designed on the basis of the expression vector
pET-46 Ek/LIC (Novagen, Germany) contained the sequence of
the phytase gene Bacillus sp. M2.11 under promoter T7. Phytase
was isolated in three steps by affinity chromatography on Ni-
beads, ion-exchange chromatography on Q-Sepharose and gel
filtration on a column of Sephade G200 16/60 via FPLC. After
the three steps of cell lysates purification we obtained phytase
preparation with purity in 50%, and an output for 9.1% of the
activity. It has to be noted that other bacillary phytases
were cleaned using chromatography on phenyl Sepharose,
DEAE-Sepharose, columns MonoS, MonoQ [Choi Y.M., Suh
H.J., Kim J.M. Purification and properties of extracellular phyt-
and characterization of a bacterial phytase whose properties
make it exceptionally useful as a feed supplement // Protein J.
2007. V. 26. P. 467–474]. Our methods of affinity chromatog-
raphy yielded chromatographically homogenous phytase prepara-
tion from the lysates of the recombinant strain. The homogeneity
of the purified enzyme was also confirmed by electrophoresis PA-
Agv 12% SDS when there was obtained only one protein band.
The molecular weight of the protein was 41 kDa. The primary
structure of the purified phytase Bacillus sp. M2.11 were deter-
dined by using MALDI-TOF-mass spectrometry. It was found
that the amino acid sequence of mature protein was identical to
the amino acid sequence of the phytase cloned into the vector
of overexpression. The amino acid sequence of the phytase included 371 amino acid residues, corresponding to a molecular
weight of 41 kDa protein and confirmed the data obtained by
SDS-electrophoresis. The molecular weight of the purified
enzyme was near by with a molecular weight of extracellular phyt-
ase B. subtilis (44 kDa) [Farhat-Khemakhem A., Farhat M.B.,
Boukhir I., Bejar W., Bouchala K., Kammoun R., Maguin E.,
Bejar S., Chouayech H. Heterologous expression and optimiza-
tion using experimental designs allowed highly efficient produc-
tion of the PHY US417 phytase in Bacillus subtilis 168//AMB
Express. 2012. V. 2. P. 10]. The isoelectric point of phytase Bacil-
lus sp. M2.11, established on the basis of the structure was pI
4.8.

Thus, we isolated and purified phytase of Bacillus sp. M2.11 from
E. coli recombinant strain. Molecular weight of the protein
was 41 kDa and pI 4.8.

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**SW06.W33–62**

**Heterologous production of penicillin G acylase (PGA) from Bacillus megaterium in Pichia pastoris**

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Penicillin G Acylase (PGA) protein (EC 3.5.1.11) is a commer-
cially valuable enzyme used in the production of semisynthetic β-
lactam antibiotics. It is responsible for the penicillin G hydrolysis
into phenylacetic acid and 6-amino penicillamic acid (6-APA),
which is the starting compound for the synthesis of many β-lac-
tam antibiotics. Penicillins are one of the most relevant antibiot-
ics used and produced in the world. Semisynthetic penicillin
exhibit many advantages, like great stability, easier absorption
and less side effects than natural ones. PGA is a heterodimeric protein synthesized as a single-polypeptide precursor that under-
goes an autocatalytic processing to remove an internal spacer
peptide to produce the active enzyme. The requirement of a com-
plex post-translational processing seems to be a limiting factor
for the expression of PGA in recombinant E. coli strains. Previ-
os studies demonstrated that the PGA protein from B. megateri-
um was expressed in E. coli system as a single polypeptide,
representing the PGA precursor preproprotein form without
activity. The methylotrophic yeast P. pastoris has advantages in the protein processing and post-translational modifications. Moreover, the expression of foreign genes is obtained by homologous recombination, the product is secreted, and the system is also manageable for industrial scale up. In this study, the PGA gene was cloned into a propagation vector (pTZ57R/T), subcloned into the pPICZαA expression vector and expressed under control of AOX1 promoter in Pichia pastoris GS115. Penicillin G acylase was expressed fused with a polyhistidine tag and purified by metal affinity chromatography from the cultured filtrate of P. pastoris. Using SDS-PAGE electrophoresis, two distinct subunits with apparent molecular weights of 25 000 Da (alpha) and 62 000 Da (beta) were found. The enzyme activity was detected in the cultured medium of the P. pastoris transformants using a method based on the formation of yellow 2-nitro-5-aminobenzoic acid by penicillin G acylase acting on 2-nitro-5-phenylacetaminobenzoic acid (NIPAB). This study is the first report of a successful clone of PGA protein from B. megaterium in P. pastoris and provides helpful information in respect to PGA production and cloning systems strategies.

**SW06.W33–63**

**Functional expression of ligand-binding domains of eukaryotic proteins in E. coli membrane: exercises with Kv1.3 channel**

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The aim of our research is to work out the principles of bioengineering systems based on presentation of ligand-binding sites of eukaryotic proteins at the surface of bacterial membrane. These systems together with fluorescent detection techniques can be used to study ligand-protein interactions and to search for novel ligands. As an example of this approach, we present here a system for search and study of Kv1.3 outer pore ligands using recombinant hybrid channels KcsA-Kv1.3 expressed in the cytoplasmic membrane of E. coli. Search for new efficient and selective blockers of potassium Kv1.3 channels is of medical importance since Kv1.3 is a promising target in the treatment of a number of autoimmune diseases and cancer. In our system, KcsA-Kv1.3 channels form ligand-binding tetramers directly in the bacterial cell membrane. Oriented location of these tetramers in the lipid bilayer with their ligand-binding sites exposed into the periplasmic space makes them accessible for fluorescently labeled ligand, for example, agitoxin-2 (R-AgTx2), which is added exogenously. High level of expression of KcsA-Kv1.3 leads to a bright specific staining with R-AgTx2 of channel-presenting spheroplasts. Ligand-protein interactions are detected at the surface of spheroplasts using laser scanning confocal microscopy. To reduce the influence of fluorescence on the detection of ligands, we used 10–50-fold lower concentration of R-AgTx2 for confocal staining. KcsA-Kv1.3 channels from E. coli with and without fluorescent ligands were purified from E. coli lysates using PEG precipitation and Ni-bearing Ni-NTA affinity chromatography, respectively. On the one hand, purified KcsA-Kv1.3 channels have a very high affinity for fluorescent ligands. On the other hand, the system provides a simple and reliable method for isolation of ligands from mixtures containing other proteins and metabolites of E. coli. Further experiments will be focused on purification of ligands from E. coli-KcsA-Kv1.3 membranes using affinity chromatography and mass spectrometry.

**SW06.W33–66**

**Phytase of Bacillus ginsengii: cloning, expression and purification**

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Phytase is used as a feed additive for degradation of antinutritional phytate. These enzymes are not produced in digestive tracts of pigs, poultry and other monogastric animals. Phytic phosphorus passes unchanged through the digestive track of the animals and comes with a litter which applies in soil. Microbial phytases split phytates and convert phosphorus and associated metal ions in condition available for animals. The maximum suitable for use phytases are still not found. Therefore, the constant search of new phytases producers is still relevant.

Bacteria showing phytase activity was isolated from soil of agricultural company of Republic of Tatarstan. Strain was identified by 16S rRNA gene sequencing as Bacillus ginsengii. Results of phytase gene’s sequence alignment presented in the international databases revealed a high degree of homology of these genes between bacillary cells. By virtue of data PCR fragment of phyB gene was obtained. Phytase gene was cloned into pET-46 plasmid by LIC system. New construction was transformed in Escherichia coli Rosetta 2 (DE3) cells. Phytase was isolated in three steps by affinity chromatography on Ni-beads, ion-exchange chromatography on Q-Sepharose and gel filtration on a
column of Sephadex G200 16/60 via FPLC. After the three steps of cell lysates purification we obtained phytase preparation with purity in 500, and an output for 9.1% of the activity. The homogeneity of the purified enzyme was also confirmed by electrophoresis PAAgV 12% SDS when there was obtained only one protein band. The molecular weight of the protein was 41 kDa. The primary structure of the purified phytase \textit{B. ginsenghumi} were determined by using MALDI-TOF analysis. It was found that the amino acid sequence of mature protein (371 amino acids) was identical to the amino acid sequence of the phytase clonned into the vector of overexpression. The isoelectric point of phytase \textit{B. ginsenghumi} was pl 4.8.

Thus, we isolated and purified phytase of \textit{B. ginsenghumi} from \textit{E. coli} recombinant strain. Molecular weight of the protein was 41 kDa and pI 4.8.

This work was supported by the federal Grant ‘Science and teaching program of innovative Russia’ for 2009–2013 year, agreement number is №14.A18.21.0575 from 10.08.2012 and grant of Russian Foundation for Basic Research12-08-00942a.

**SW06.W33–67**

The study of molecular mechanisms of antioxidant action of bile pigments and investigating their role during the interaction of biomolecules with a drug carrier protein

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Long period, bilirubin, the main product of oxidation hem-containing proteins, bile pigment was considered to be as ballast product of its metabolism and toxic agent. However, it was found that bilirubin is able to inhibit free radical reactions, but this fact has long remained overlooked. It is thought that the normal physiological concentration of bilirubin in the tissues and organs are now considered to be vital. The antioxidant function of bilirubin clinically indicated in a variety of pathologies such as ischemia-reperfusion injury, atherosclerosis, hemorrhagic stroke, anaphylactic reactions, chemical mutagenesis. These findings can lay the foundation of new treatments for cardiac, cancer and neurodegenerative diseases. However, the detailed mechanism of bilirubin antioxidant function is still remains unknown. The solution of this problem is particularly relevant for understanding the pathogenesis of bile pigments and development of new therapeutic treatments for a number of common hyperbilirubinemia diseases.

This study aims to carry out the multivariate analysis of oxidative reactions of bilirubin and to build multivariate models antioxidant and antiradical activity of bile pigments, and to establish its role in the non-covalent binding of various biologically active compounds (such as RNA and DNA bases, BODIPY fluorescent probes and others) to protein carriers. At present time we have established the mechanisms and kinetics of bilirubin oxidation at different conditions. It was found that bilirubin is an inhibitor ascorbic acid oxidation which is powerful antioxidant.

Considering also that bilirubin forms a macromolecular complex with transport protein albumin in organism, current research aims to establish the binding parameters of various biologically active compounds to the protein. This approach can be a basis for development of advanced test systems to determine the type and capacity of small molecules to interact with proteins, as well as in drug design and molecular recognition. The proposed solution on the background of the active work of biologists and physicians will bridge the gap of research in the field of chemistry of bilirubin and its analogs.

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**SW06.W33–67**

Metabolic engineering of cephalosporin C producer – \textit{Acremonium chrysogenum}:
overexpression of MFS transporter CefT changes biosynthesis profile of beta-lactam compounds

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\textit{Acremonium chrysogenum} is a filamentous fungus, the natural producer of beta-lactam antibiotic cephalosporin C (CPC) – the starting compound for production of semisynthetic cephalosporin antibiotics.

CPC biosynthesis is the complex and highly compartmentalized process subject to multiple layers of control. Precursors’ uptake, transport of intermediates, CPC export are regulatory processes, which influence the overall CPC production. Overexpression of \textit{cefT} gene, located in the ‘early cluster’ of CPC biosynthesis genes, in low-producing \textit{A. chrysogenum} strains stimulates cephalosporsins production. CefT is an MFS-transporter belonging to the drug H\textsuperscript{+}-antiporter family. It was suggested that CefT is a possible specific CPC-carrier. However, \textit{cefT} knock-out strains are still able of CPC production, so the function of this protein is not clear.

To get more insight in the role of \textit{cefT} the effect of constitutive \textit{cefT} overexpression on the rate and ‘quality’ of CPC production in high-producing strain \textit{A. chrysogenum} VKM F-4081D was studied. A model system \textit{S. cerevisiae} was used to investigate the functional activity and subcellular organization of constructed CefT-TagCFP hybrid. We have shown, that CefT-TagCFP localizes in proper manner in yeast plasma membrane. Expression of this protein in \textit{S. cerevisiae} MFS deletion strains confers resistance to spemidine, ethidum bromide, hygromycin B, complementing functions of orthologous MFS transporter genes \textit{qfr3}, \textit{gpo1}, \textit{gpo3}.

Recombinant \textit{A. chrysogenum} VKM F-4081D strains contained \textit{cefT} under control of the constitutive \textit{A. nidulans} \textit{gpdA} promoter. The level of \textit{cefT} expression judged by RT-PCR analysis was increased 2–9 fold as compared to parental strain. Constitutive \textit{cefT} expression resulted in significant changes in the profile of exported beta-lactam compounds. The level of deacetyl- and deacetoxy-CPC was increased, CPC yield was decreased 25–30% as compared to parental strain.

It was shown, that CefT has a broad substrate specificity and exports not only CPC, but also its intermediates. Constitutive overexpression of \textit{cefT} is not optimal strategy for increasing the productivity of industrial \textit{A. chrysogenum} strains, it leads to the ‘leakage’ of CPC intermediates in the culture medium, reducing the final CPC yield.
SW06.W33–68
Optimization of defined medium and nitrogen source for recombinant penicillin G acylase (PGA) production in *Bacillus megaterium*

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Penicillin G acylases (PGA, penicillin G amidase, E.C. 3.5.1.11) are industrially important enzymes which hydrolyze penicillin G to phenylacetic acid and 6-aminopenicillanic acid, a key intermediate for the synthesis of semisynthetic β-lactam antibiotics. In this study, a recombinant strain *Bacillus megaterium* PV361 was used to produce Penicillin G Acylase (PGA). The effect of the concentration of metal ions in minimal media has been shown to be very important for the production and secretion of the PGA in *Bacillus megaterium* PV361. The best media compositions for biomass and PGA production were used evaluating a genetic algorithm. The screening was carried out in 96 microtube deep well plates with 900 μl cultivation volume, 4 g/l of glucose as carbon source, starting as a minimal medium and studying the concentration of 12 defined components: MgCl₂, MnCl₂, FeSO₄, CaCl₂, NH₄Cl, K₂HPO₄, H₃BO₃, CuSO₄, ZnSO₄, (NH₄)₆Mo₇O₂₄, CoCl₂ and KCl. In seven generations, 240 different kinds of media were tested, key elements for production and secretion were detected and a 10-fold increase in PGA production and 5-fold increase in biomass compared to the previously used minimal medium could be achieved. From literature and data derived experimentally, CaCl₂ and CuSO₄ were shown to be key components of the metal ions tested for biomass formation and especially for production and secretion of PGA. It was scaled-up from 96 microtube deep well plates to shaker flasks enhancing 3-fold in PGA production and 1.8-fold in biomass. With this defined medium results, it was studied the nitrogen source using some different complex components as tryptone, yeast extract, hydrolyzed casein and soytone, using 5 g/l of NH₄Cl as starting nitrogen source. The best results it was found with hydrolyzed casein enhancing 2-fold in PGA production and 1.7-fold in biomass comparing with the found defined medium. The production of PGA increased to 6480 U/l and 5.25 gDCW/l which was more than 6-fold in PGA production and 2.6-fold in biomass of that obtained in shaker flasks comparing with the minimal medium.

SW06.W33–69
Biochemical properties of intracellular laccase produced by *Sinorhizobium melliloti* strain originated from Poland

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Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyse the one-electron oxidation of variety aromatic compounds. The use of oxygen (a non-limiting electron acceptor) by the laccases makes these enzymes adequate for industrial and environmental applications e.g.: bioethanol production, dye decolorization, detoxification of industrial effluents, bioremediation, biobleaching of paper pulp, biosensing and wine stabilization. Due to the numerous biotechnological applications of laccase, it is essential to determine its biochemical properties including the effect of various physicochemical agents on enzyme activity and stability.

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The main goal of this work was to contribute to a better knowledge of these enzymes with respect to potential biotechnological application of laccases. In the present work, complete biochemical characterization of native laccase was performed.

Substrate specificity was measured using classical laccase substrates and the highest KM values were obtained for guaiacol (KM = 212 μM) and 2,6-dimethoxyphenol (KM = 342 μM). The purified enzyme was identified as a glycoprotein. SDS-PAGE and gel filtration methods were applied to determine protein molecular weight and isoelectric point. The enzyme was stable at pH 4.0 to 8.0, and the optimum pH for enzyme activity varied from 2.2 to 6.0 for ABTS and ferulic acid, respectively. The temperature optimum was 80°C. Enzyme was stable up to 7 days at 4°C, and its temperature half-life (T½) was 6 h in 30°C. Laccase activity was increased by the addition of SDS and strong inhibited by sodium azide, fluoride (electron flow inhibitor) and dithiothreitol.

The research was partially supported by the grant PSPB-079/2010 under the framework of Polish – Swiss Research Programme.

SW06.W33–70
Purification and characterization of prickly pear (*Opuntia ficus-indica*) alpha-galactosidase by three-phase partitioning

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α-Galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) are a common class of hydrolases which are widely distributed in nature. They have been isolated and purified with different techniques from various sources, such as plants, animals and microorganisms. These enzymes catalyse the hydrolysis of α-D-galactose bonds which found in galactooligosaccharides and polysaccharides. They are very useful enzymes, especially for elucidation of the biological functions of complex carbohydrates, for organic synthesis, for structural analysis, for medical purpose and in sugar industry.

Three phase partitioning (TPP) is a simple and often one-step procedure successfully used for separation and purification of enzymes and proteins in recent years. It involves the addition of a salt to the aqueous solution containing proteins followed by the addition of a water miscible aliphatic alcohol. In less than an hour three phases are formed. The upper solvent phase containing pigments, lipids, hyrophobic materials is separated from lower aqueous phase containing proteins, saccharides and cell debris by an intermediate layer. This protein-rich middle layer generally contains desired enzymes or proteins. It is also simple, inexpensive, scalable and rapid procedure that works at room temperature in comparison to conventional separation and purification processes. TPP can also be used directly with the crude suspensions. Besides of the physical conditions of the assay, the partitioning process is also affected by the hydrophilicity, the molecular weight and pI of protein.

In the present study, we have used three-phase partitioning for direct one-step purification of α-galactosidase from prickly pear (*Opuntia ficus-indica*) fruit. α-D-Galactosidase was first isolated from prickly pear with conventional protein extraction methods and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentrated and purified with TPP. TPP systems were prepared by using ammonium sulfate as a cosmotropic salt and then concentra
and substrate concentration) affecting to the enzyme activity and stability were investigated. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was also performed to determine the purity and molecular weight of the enzyme. The results showed that, with necessary optimization TPP is a simple, quick, economical and very attractive bioseparation technique for primary purification of α-galactosidasesthe compared to conventional chromatographic protocols. Biochemical properties of the purified enzyme have also indicate that, enzyme should be found a potential for its industrial applications.

SW06.W33–71
Immobilization and characterization of tomato alpha-galactosidase on Sepabead EC-EP
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α-Galactosidases (Mellibiase, EC 3.2.1.22) are the exoglycosidases that catalyse the hydrolysis of α-1,6-galactose bonded D-galactosyl residues of basic and complex oligo- and polysaccharides (raffinose, stachyose, mellibiose, galactomannans). They have been purified with different techniques from plants, animals and various microorganisms. They have great importance in especially sugar industry, elucidation of the biological functions of complex carbohydrates, structural analysis, organic synthesis and medical purpose.

Immmobilized enzymes are the heart of innovative biotechnological processes as alternatives to traditional chemical technologies. For the industrial development of biocatalytic processes an effective immobilization method is commonly required to allow the reuse of enzymes or continuous processing. Covalent immobilization has the advantage of forming strong and stable linkages between the enzyme and the carrier that result in robust biocatalysts. Immobilized enzymes may also exhibit much better functional properties than the corresponding soluble enzymes by very simple immobilization protocols. Epoxy-activated supports have been proposed as very efficient materials for the immobilization of enzymes at the industrial scale for different reasons: e.g. high stability of the groups at neutral pH values even in wet conditions, commercial supports can be stored for long periods of time, high stability of the enzyme-support bond, possibility of performing a final blocking of the remaining groups, possibility of achieving stabilization of the enzymes via multipoint covalent attachment. Sepabeads EC are polymethacrylate-based carriers and can be successfully used for the immobilization of enzymes. The series Sepabead EC-EP are epoxy activated, with high reactive group density. The chemistry for attachment of the enzyme to the support is straightforward. Compared with other epoxy acrylic polymers, Sepabeads EC-EP possess a high mechanical stability, high resistance to microbial attack and do not swell in water. They are particularly suitable for covalent immobilization of enzymes for industrial applications because of their excellent mechanical properties when used in bioreactors.

In the present work, α-galactosidase was isolated and partially purified from tomato (Lycopersicum esculentum) and then immobilized on Sepabead EC-EP. We have aimed to prepare an immobilized α-galactosidase giving the best combination of remaining activity and long-term stability. For the optimization of immobilization protocol, the effects of some parameters (pH, ionic strength, enzyme amount etc.) to enzyme immobilization were investigated. Some biochemical properties of the immobilized enzyme regarding remaining activity, storage, operational, pH and thermal stability as well as the influence of the temperature and pH on the activity of the immobilized enzyme were determined. The results showed that tomato α-galactosidase was successfully immobilized on Sepabead EC-EP. The immobilization procedure on this support is very cheap and easy to carry out. The good properties of the immobilized enzyme offer potential for use in various production processes.

SW06.W33–72
 Biosynthesis and isolation of STEAP1 peptides for prostate cancer immunotherapy
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Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is located at cell membrane and it is over-expressed in prostate cancer. Several studies have been suggested STEAP1 as a putative target for immunotherapy or as a target to deliver drugs in cancer cells [1]. Hence, it becomes crucial to identify and characterize the affinity between commercial antibodies and STEAP1 protein. In an attempt to identify the STEAP1 region that presents higher affinity to target antibody, three different peptides were biosynthesized, namely extracellular, transmembrane, or intracellular region. To produce these peptides, the DNA sequences were amplified by PCR using specific primers containing an affinity tag of histidines. Subsequently, PCR products were cloned into pUC19 vector expression, selected in Escherichia coli (E. coli), and plasmid DNA was sequenced in order to confirm the identity of DNA cloned. Once the desired DNA cloning was obtained, the three peptides were expressed in E. coli cells and growth in a standard complex medium at 37°C. 250 rpm for approximately 8 h, followed by a freeze/thaw lysis. In practice, affinity tag allows the purification of any peptide, and become an emerging and powerful tool. According to this reasoning, an immobilized metal chelating chromatography (IMAC) technique with Ni²⁺ was implemented and tested with different buffers, which differ in composition and concentration (sodium chloride, sodium phosphate, imidazole and urea). Our results suggest that this strategy could be helpful to produce and purify peptides with low molecular weight in order to analyze the kinetic affinity between antibody/antigen in a biocore system.

Reference

SW06.W33–73
Fabrics made from genetically modified flax enriched in polyhydroxybutyrate as an effective dressing for long-standing wounds
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Flax is a source of many valuable components with significant potential for biotechnological application. To increase the valuable qualities of flax products, the flax genome has been genetically modified. One of such modification was made by introducing polyhydroxybutyrate (PHB) synthesis genes into flax genome resulting in fibers with much improved biomechanical properties and phenylpropanoid content. The flax fibres contain very little of a soluble phenolics, those mainly being ferulic and coumaric acids and vanillin and apigenin C-glucosides. The majority of the phenolic lopropanoid content. The flax fibres contain very little of a soluble phenolics, those mainly being ferulic and coumaric acids and vanillin and apigenin C-glucosides. The majority of the phenolic a composite and wound dressing production. Unprocessed
flax fabrics affect skin cell proliferation in a dose dependent manner. The PHB containing fabric promotes the proliferation of keratinocytes and fibroblasts, reduces human monocyte cell line proliferation and activates human dermal microvascular cell migration. The aqueous extract from fibres has a modulatory effect on the free radical level in monocyte cells, and it scavenges the excess level of ROS in activated cells. Incubating the cells with the flax fabrics prior to H$_2$O$_2$ treatment also diminished the amount of DNA damage, as established using the comet assay and pulsed field electrophoresis of intact cellular DNA.

Based on those fibres a LenplastBis – the wound dressing with anti-inflammatory and antibacterial function was developed. In pre-clinical trials it was observed to stimulate granulation and epithelialization resulting in significant reduction in a wound size. The treatment is divided into three stages – first consisting of a flax fabric made from genetically modified flax, second – with flax fabric wetted with oil emulsion and third - flax fabric wetted with a seed-cake preparation. Due to a content of a antibacterial components it can be used also on a moderately infected wounds.

**SW06.W33–74**

**The Cerrena unicolor laccase overproduction on waste agricultural based media**

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Laccases (EC 1.10.3.2, $p$-diphenol: oxygen oxidoreductase) are multi copper-containing oxidases, catalyzing the oxidation of ortho- and para- diphenols, polyphenols, arylamines, aminophenols and some inorganic ions, while simultaneously reducing molecular oxygen to water. In the fungi belongs to the most effective producers of this biocatalyser were discovered three forms of laccases so called blue [1], yellow [2] and white ones [3]. Because of their broad substrate specificity, native or immobilized laccases can be potentially used in many industrial applications [4].

Recently, *Cerrena unicolor*, was determined as a new fungal source of extracellular laccase, excreting the enzyme under non-induced conditions with a rate similar to the best laccase producers [5]. Reducing the costs of laccase production by optimising the fermentation process by finding efficient and cheap carbon source in the media is the basic research for the industrial applications [6].

For these reasons the present study was conducted in order to find the best cultural conditions for the overproduction of laccase by *C. unicolor* in shaken flasks and aerated fermenter cultures, as well as in the fluidized bad reactor. Additionally the search for the most effective inducers of enzyme synthesis in the media containing the agricultural waste substances was done.

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**References**


**SW06.W33–75**

**Bioinformatic analysis and molecular modeling reveal mutation bD484N to stabilize penicillin acylase and improve its catalytic performance in alkaline medium**

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Rational design strategy based on bioinformatic analysis of Ntn-hydrolase family enzymes and molecular modeling of potential mutants was applied to improve stability and catalytic performance of penicillin acylase from *Escherichia coli*. Recently developed method of bioinformatic analysis [1–3] and corresponding computer program Zebra [http://biokinet.belozersky.msu.ru/zebra] were applied to identify subfamily-specific positions that were supposed to be responsible for discrimination of functional properties of Ntn-hydrolases and considered as hotspots for rational design of penicillin acylase mutants. Molecular dynamics was used to simulate pH-dependent inactivation of wild type enzyme and its mutants. Subfamily-specific position bD484 was identified as a key element of the buried side chain interaction network, which collapse at alkaline pH disturbs a native protein conformation. This crucial residue was chosen as a hotspot for mutation to engineer enzyme variant stable in alkaline medium: single stabilizing substitution bD484N has been proposed by bioinformatic analysis of homologous Ntn-hydrolases with different pH stability. The bD484N mutant was expressed, purified and characterized experimentally. The bD484N mutation substantially stabilized penicillin acylase in alkaline medium (10-fold improvement at pH 10.0) however even more important was enzyme stabilization to inactivation at high substrate concentration [4]. Observed stabilization effects allowed to improve catalytic performance of penicillin acylase at enzymatic peptide synthesis in aqueous medium (where due to the high pKa values of the amino group of free amino acids alkaline pH should be used in order to have external nucleophiles in their reactive form) and leaded to five-fold increased yield of preparative N-phenylglycine-derived peptide synthesis from equimolar substrate mixtures compared to the wild type enzyme.

**Reference**

SW06.W33–76
Modulating electron transfer in cytochrome P450 3A4 fusion enzymes
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Cytochromes P450 (P450s) are haem thiolate enzymes that catalyse the NADPH-dependent monooxygenation of different xenobiotics including drugs. The function of the mammalian P450s relies on a redox chain that involves a reductase flavo-protein that mediates the transfer of electrons from NADPH to the haem catalytic center. In recent years, much attention has been devoted to the creation of fusion systems comprising both the catalytic and reductase components [1].

The genes encoding for the human cytochrome P450 3A4 and the soluble reductase domain of P450 102A1 from Bacillus megaterium (BMR) have been joined by a short peptide linker (Ser-Pro-Arg) and successfully expressed in E. coli by our group [2]. In order to better understand the mechanism of electron transfer (ET), two constructs (3A4/BMR-3Gly and 3A4/BMR-5Gly) were also engineered varying the length of the linker connecting the haem and the reductase domains. Initially the rate of NADPH consumption by the three different chimeric proteins was measured. The results obtained showed a faster consumption by 3A4/BMR-3Gly compared to the other two proteins, 2.8 ± 0.2 ms vs 1.4 ± 0.1 ms. Subsequently, flavin-to-haem electron transfer rates were measured using stopped-flow as the increase in absorbance at 450 nm in the presence of NADPH and substrate after addition of carbon monoxide. Also in this case, 3A4/BMR-3Gly showed a faster rate of 2.2 ± 0.4 ms compared to 0.5 ± 0.1 ms for the other two chimeric proteins. It is interesting to note that the two chimeras with the slow ET show an initial lag phase of several seconds probably due to the rearrangement of the proteins for obtaining the best ET complex.

These results demonstrate that the length of the linker is an important factor in modulating the ET between the reductase and the haem domain within the P450 fusions.

References
A novel like expression system for production of applied Bacillus proteolitic enzymesprint

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For the production of various industrial proteins the expression systems are the most powerful approach. In this work we develop a novel heterologous LIKE expression system based on the promoter of the liaIH operon under control of the LiaR-LiaS two-component system Bacillus subtilis. Phial promoter responses to stress caused by cell wall antibiotics. The LIKE protein expression system offers a number of advantages over existing systems: (i) a tightly switched-off promoter during exponential growth in the absence of a stimulus, (ii) a concentration-dependent activation of Phial in the presence of suitable inducers (iii), a very fast but transient response with a very high dynamic range of over 100-fold (up to 1000-fold) induction, (iv) a choice from a range of well-defined, commercially available, and affordable inducers, (v) the option to develop food-grade expression strains, and (vi) the convenient conversion of LIKE-derived inducible expression strains into strong constitutive protein production factories. B. subtilis LIKE system has proven to be useful tool for reporterlac Z and gfp genes. However, compared to the production level of the intracellular proteins, the amount of extracellularly produced heterologous serine proteases was lower. Consequently, in this work, a directed optimization of the antibiotic-inducible protein export system was carried out. To achieve this goal three signal peptides (SPYngK, SPAsp, SPPac) from B. megaterium facilitated the secretion of the Thermobifida fusca heterologous hydro-lase (Tfh) were cloned in single copy (stably integrated into the chromosome) and on a multi-copy LIKE plasmids. Now we evaluate capability to promote protein secretion of the heterologous proteases (AprBp, GseBp and MprBp) from B. pumilus with selected signal peptides in a part of LIKE expression system. B. pumilus proteases are of interest for practical medicine, since they possess high fibrinolytic, thrombolytic and anticoagulant properties. There is a tendency for increased two directions: the creation of drugs that stimulate fibrinolysis activators with fibrinolytic systems of the body and drugs that reduce coagulant activity of platelets. Therefore we also investigated the ability of serine proteases to lyse clots (thrombolytic activity) and to prevent the formation of blood clots (anticoagulant activity). We detected that all enzymes have fibrinolytic activity by the fibrin plate method. In vitro experiments showed that proteases have a pronounced anticoagulant effect. It is concluded that the serine proteases of B. pumilus have high fibrinolytic activity, and thrombolytic and anticoagulant properties. These results suggest that the subtilisin like proteinase (AprBp) and glutamyl endopeptidase (GseBp) of B. pumilus are perspective for the development of thrombolytic drugs. Moreover, the investigated enzymes are not toxic to the gram-positive and gram-negative bacteria in any of the tested concentrations. During the micronucleus test clastogene effect also was not observed. However, serine proteinases of Bacillus were able to exert cytotoxic effects on an immortalized cell lines of animals, but not always the destruction of the cell monolayer was accompanied with cell death.

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Immiscibility of Carbonic anhydrase II (CAII) enzyme on superparamagnetic iron-oxide nanoparticles

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Carbonic anhydrase (CA), a zinc metalloenzyme, catalyzes the conversion of carbon dioxide to bicarbonates and/or vice versa. Some reports entail that Carbonic anhydrase (CA) can be effectively used for the sequestration of CO₂. Therefore, immobilization of CA may be the beginning of one of the viable and environmental friendly process which may reduce the size of the reactor.

In this work, recombinant human CAII was expressed in E. coli BL21 cells and purified using affinity chromatography and magnetic iron oxide nanoparticles were prepared by co-precipitation Fe⁺² and Fe⁺³ ions in an ammonia solution at room temperature. X-ray diffraction (XRD) and the magnetic measurements showed that the nanoparticles are magnetic and superparamagnetic, respectively. Saturation magnetizations, M, of the CAII bounded nanoparticles after the immobilization and after the enzyme activity ended are 50.58 and 48.62 emu/g, respectively. Free CAII enzyme and CAII bounded iron oxide nanoparticles were characterized by fourier transform infrared spectroscopy. The enzymatic measurements indicated the CAII-bound nanoparticles retained 22% of its original activity.

SW06.W33–80
Optical control of cell physiology using genetically encoded fluorophores

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The most promising research field is an investigation of living systems on different level of organization by using optical bioimaging techniques and genetically encoded fluorophores (fluorescent proteins). FP may play role as a markers, photosensitizers, sensors and permit to estimate the cell physiology on the subcellular level and processes in a whole body. For purposes of optical control different techniques can be useful.

Fluorescence diffuse tomography (FDT) allows performing 3D reconstruction of a fluorophore distribution inside high scattering biological object of several centimeters in size. This technique has demonstrated its high potential for in vivo imaging of small animals bearing tumors labeled with different fluorophores. There are several promising directions of fluorescent imaging evolution, one of them is the whole-body time-resolved fluorescence imaging which allows one to estimate lifetime of the fluorophore. The use of fluorescent proteins as tumor markers enables an accurate tumor location and estimation of the tumor growth by FDT.

Optoacoustic tomography (OAT) is based on the 3D reconstruction of an internal distribution of absorption coefficient by precise measurement of ultrasonic waves which are generated due to partial conversion of absorbed energy from external pulsed optical radiation into heat. The possibility of OAT to control photobleaching of the fluorophores in real-time may be very...
helpful in development of photodynamic therapy with genetically encoded phototoxic proteins.

*Laser scanning microscopy (LSM)* is a gold standard for imaging fluorescent biological objects in vitro with subcellular spatial resolution. Two-photon fluorescence microscopy (TPFM) provides extended abilities for deep microscopic imaging of highly scattering biological objects in vivo. For biomedical applications TPFM is highly advantageous due to low average-power radiation, reduced fluorophore photobleaching and the ability of long-term observation with high contrast and resolution. The utmost information out of cells and tissue can be additionally obtained by spectral resolved fluorescence lifetime imaging (sFLIM). Breaking diffraction limit by novel techniques like Stimulated Emission Depletion (STED), stochastic optical reconstruction microscopy (STORM) reveals outstanding possibilities for nano-scale bioimaging. As the fluorescent proteins can be genetically targeted to any compartment of the cell, all these technologies widely use fluorescent proteins for marking of the different cell organelles.

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**Recombinant analogs of spider silk proteins as the base of new biomaterials for medical applications**

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The unique properties of spider dragline silk (combination of high mechanical strength and extensibility resulting in a very high toughness, stability at a wide range of temperatures, solubility in organic solvents, high biocompatibility and slow biodegradability) provide a basis for a variety of biomedical applications. It has a higher tensile strength and is stiffer, more extensible and less immunogenic than the commonly used native silkworm silk. In spite of this dragline silk has not been produced commercially to date. Aiming at the development of recombinant analogs dragline silk proteins we have designed using computer analysis of existing databases and mathematical modeling, synthesized and cloned in yeast cells two analogs of spidroins 1 and 2, proteins that make up the dragline silk. Both proteins were expressed in *Sacharomices cerevisiae* and purified using a cation exchange chromatography. The method of the proteins isolation and purification is very simple and can be easily scaled up. For various biomedical applications, both spidroins can be processed into a variety of structural forms, including transparent nanoporous films, highly porous electrospun films, thin fibers, hydrogels, including microgels, microbeads, and sponge-like matrices. Comparative study of two porous scaffolds based on silk fibroin of *Bombyx mori* and a recombinant spidroins showed that the vascularization and intergrowth of the newborn connective tissue with nerve fibers, at 8 weeks after subcutaneous implantation in mice was more profound in the case of spidroin scaffolds. Implantation of both scaffolds into bone defects in rats accelerated repair compared to controls and regeneration was determined to be more effective with spidroin scaffolds. The unique property of spidroin matrices – the spontaneous formation of micropores of 2-15 mm in diameter in the walls. To improve the cell attachment we introduced RGD-like sequences into recombinant analogs of spidroins by gene-engineering method. The modification resulted in scaffolds, films and microgels 2-fold increase in binding to cells, and a significant increase in cell proliferation. The results indicate the great potential of the new material for tissue regeneration, wound healing and other medical applications.