Young Scientist Forum

YSF–1
Structural basis of a plant photosystem I sunlight conversion

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A plant Photosystem I (PSI) is a large membrane super-complex that drives photosynthesis. PSI captures sunlight through sophisticated pigment network and uses the energy to perform transmembrane electron transfer. It consists of the reaction center complex (RC), where the charge separation reaction takes place and the light harvesting complex (LHCI), which serves as an additional antenna system. PSI performs a photochemical activity with the unprecedented quantum yield of close to 100%, being the most efficient light capturing and energy conversion machine. We determined the X-ray crystal structure of the intact PSI from plants at 3.4 Å resolution (1,2). The current crystal structure provides a picture at near atomic detail of 17 protein subunits and shows how the biological significance of plant PSI is matched by its structural elements. 3038 amino acids were assigned, as well as 168 chlorophylls, two phyloquinones, three Fe_4S_4 clusters and five carotenoids. The final model consists of not less than 45 transmembrane helices and represents one of the most complicated membrane complexes for which a near atomic model was determined. The remarkable feature of PSI is the unprecedented high content of non-protein components – approximately one third of the total mass of about 600 kDa consists of different cofactors. The structure reveals intriguing insights regarding unique interactions between the RC and the LHCI complexes and provides a structural basis for the state transitions phenomenon. In addition, putative docking sites of the soluble electron carriers are described for the first time.

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YSF–2
Intermedin, a novel peptide, induces coronary microvascular endothelial barrier failure via RhoA/Rock-dependent derangement of actin cytoskeleton

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Intermedin (IMD) is a novel member of adrenomedullin peptide family which has been shown to be released by endothelial cells (EC) and acts via increased production of cAMP via adrenomedullin receptors coupled to adenylyl cyclase. Recently we have shown that maneuvers increasing intracellular levels of cAMP stabilize macrovascular aortic EC barrier but in contrast induce failure of barrier function in microvascular coronary EC. Therefore, here the effect of IMD on endothelial barrier function of coronary microvascular EC was studied.

Methods and Results: In cultured rat coronary microvascular EC monolayers IMD (10 nM) increased permeability (albumin flux), caused inactivation of small GTPases, RhoA and Rac1 (pull-down assay), the key regulators of EC cytoskeleton. This inhibition was accompanied by dissassembly of actin filaments (confocal microscopy), dephosphorylation of paxillin (western blot) and loss of focal adhesions and VE-cadherin from cell-cell adhesions leading to rapid stellation of EC. These IMD effects were partially blocked by a calcitonin receptor like receptor (CRLR) inhibitory peptide (CGRP8-37; 1 M) and a PKA inhibitor (H89; 1 M). Accordingly, forskolin, a direct activator of adenylyl cyclase, and a cAMP-analog 6-Bnz-cAMP, a specific direct activator of PKA mimicked these IMD effects, further strengthening PKA-mediated effects of IMD. Inhibition of RhoA by C3 transferase (2 μg/ml) and a specific Rock inhibitor Y27632 (10 nM) produced similar effects. Inhibition of protein tyrosine phosphatases (PTPs) with orthovanadate (500 μM) abolished IMD (as well as RhoA/Rock inhibition)-induced EC barrier failure, Rac1 inactivation as well as paxillin dephosphorylation, but had no effect on RhoA inactivation. Inhibition of PTPs lead to activation of Rac1 and the rearrangement of actin cytoskeleton at cell periphery and translocation of VE-cadherin and focal adhesions at cell-cell adhesions.

Conclusion: The data of present study demonstrate that in coronary microvascular endothelial cells, IMD induces barrier failure, mainly by inactivation of Rac1, RhoA, and dephosphorylation of paxillin. This leads to dissassembly of actin filaments, and inactivation of focal adhesion.

YSF–3
Metabolism and nuclear receptors activation by ochratoxin A in primary cultures of human hepatocytes

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Background: Ochratoxin A (OTA) is a mycotoxin produced by fungi of two genera: *Penicillium* and *Aspergillus*. OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to several species of animals and to cause kidney and liver tumours in mice and rats. Biotransformation of OTA has not been entirely elucidated. At present, data regarding OTA metabolism are controversial. Several metabolites have been characterized *in vitro* and/or *in vivo*, whereas other metabolites remain to be characterized. Several major mechanisms have been shown as involved in the toxicity of OTA: inhibition of protein synthesis, promotion of membrane peroxidation, disruption of calcium homeostasis, inhibition of mitochondrial respiration and DNA damage. The contribution of metabolites in OTA genotoxicity and carcinogenicity is still unclear.

Objective: The aim of this study was to investigate the cytochroms P450 induced by OTA in human cultured hepatocytes and to determine if OTA can activate nuclear receptors, pregnane X receptor (PXR), constitutive androstane receptor (CAR) and the Aryl hydrocarbon Receptor (AhR).

Methods: We looked, firstly, on mRNA expression of some cytochroms known as target genes of these receptors and then, on receptors mRNA level using real-time quantitative reverse transcription-polymerase chain reaction (RT-QPCR).

Results: Our results showed for the first time that, treatment of primary cultured hepatocytes with increasing concentrations of OTA for 24 h, caused a significant up-regulation of CYP3A4, CYP2B6 and in a lesser extent CYP3A5 and CYP2C9, while, PXR and CAR mRNA expression were not affected. OTA was found also to induce an over expression of CYP1A1 and CYP1A2 accompanied by an increase in AhR mRNA expression. These findings suggest that these nuclear receptors could be involved in metabolic activation and toxicity mediated by OTA.

Conclusions: Our results support the presence of new transduction pathways, the PXR and/or CAR and AhR pathway. Both the PXR and/or CAR and the AhR pathways are activated by OTA

within a similar range of concentrations. The observations raise the question of OTA toxicity in the liver, the major side of expression of the promiscuous human PXR. More attention needs to be paid to effects of OTA in reproductive organs that mainly express AhR and ER. Further studies will be needed to recognize the molecular mechanism of interaction of OTA with nuclear receptors.

YSF–4
Active and directly expression of collagen, stem cells and angiogenesis in rats dermal wound healing

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In mature skin, wound repair typically begin with hemostasis and inflammation. This is followed by a proliferative phase with re-epithelialization, angiogenesis and collagen production and end with the generation of permanent scar. We have recently reported that the healing of burn injuries (second-degree burns in 20% of their body) from five groups of Wistar rats was significantly accelerated by using lipid extracts from two mussels *Mytilus galloprovincialis* Lmk (Mediterranean mussel) and *Rapana venosa* (hard shell clam) (1). In this work, the relationship between the expressions of collagen IV, CD34 and CD117 antibodies were studied by immunohistochemistry staining to further unravel the mechanism under the healing process of the different treatments employed. The immunostaining carried out in small blood vessels and capillaries of granulation tissue of the dermis, endothelial membrane, fibroblasts, basal and stem cells was different for all five groups of Wistar rats, showing the major immunopositive reaction for rats treated with the *Mytilus galloprovincialis* Lmk. lipid extract (Group 2). According to the obtained results, as expressed by histological studies, the most abundant blood vessels, collagen fibres, basal and stem cells were found only in Group 2, in good agreement with our previously reported results. Based on these results, we envisage a more widespread use of the extracts in therapeutic dermal treatments as well as in future advances on regenerative skin care ingredients.

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YSF–5
Equinatoxin, a eukaryotic pore-forming toxin used as a specific marker for cellular sphingomyelin

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Most cellular sphingomyelin (SM) resides in the outer leaflet of the plasma membrane but is synthesized *de novo* by a SM synthase1 in the Golgi complex. SM is not only important constituent of membrane, but it also serves as a reservoir for lipid signaling molecules. SM is synthesized in the lumen of Golgi and is not exposed to the cytosol, according to the published data. Equinatoxin II (EqtII), a eukaryotic pore-forming toxin from the sea anemone *Actinia equina*, shows sphingomyelin (SM) dependent activity. EqtII specifically binds SM, but not other lipids, such as cholesterol, phosphatidylcholine or other sphingolipids. Residues Trp^{112} and Tyr^{113}, both located in the membrane interacting region of EqtII, are responsible for the binding and recognition of a single SM molecule. Alanine mutants (W112A, Y113A) do not bind SM, while a protein with Trp^{112} mutated to Leu (W112L) exhibits similar lipid binding specificity to the wild type EqtII. We used EqtII fused to GFP (Eqt-GFP) as a new SM-specific marker, to obtain information on the cellular distribution of SM. Purified Eqt-GFP, expressed in *Escherichia coli*, shows SM dependence as the wild-type EqtII and binds to the cellular membrane when added to Madin-Darby canine kidney II (MDCK) cells from the outside. Images obtained with confocal microscopy on MDCK cells transfected with Eqt-GFP in a pcDNA3.1/CT-GFP shuttle vector show spotted perinuclear distribution. Mutants W112A and Y113A mutants of Eqt-GFP and Dr1, a nonlytic EqtII homologue from zebrafish show different distribution than the Eqt-GFP, while W112L shows similar distribution. The EqtII-GFP strongly co-localizes with the Golgi complex-specific marker, BODIPY-TR ceramide, while markers for plasma membrane, endoplasmic reticulum, nucleus or mitochondrium were not co-localised. These results were confirmed by subcellular centrifugation and analysis of obtained fractions by Western blots or enzymatic assays. Eqt-GFP was detected in fractions enriched with *cis*-Golgi compartments, detected with antibodies against GM130. The results collectively indicate that SM in Golgi is exposed to the cytosol. They also show the feasability of EqtII as a marker for cellular SM.

YSF–6
Transcriptional and post-translational regulation of clusterin/apolipoprotein J by the two main cellular proteolytic pathways

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Clusterin/Apolipoprotein J (CLU) is a secreted glycoprotein associated with many severe physiological disturbances that represent states of increased oxidative stress, such as aging, cancer, atherosclerosis, diabetes, renal and neurodegenerative diseases. The aim of our work was to examine the effect of proteasome and lysosome inhibition on CLU expression and to determine whether those proteolytic pathways are implicated in CLU gene regulation and protein degradation. To this end we used two different model systems, namely the U-2 OS osteosarcoma cell line and the WI38 primary human embryonic lung fibroblasts. We report that proteasome inhibition promotes both heat-shock factor 1 (HSF-1)-dependent CLU gene expression induction and protein accumulation due to reduced degradation. In contrast, lysosome inhibition results in elevated levels of CLU protein but does not affect the CLU mRNA levels. We also provide direct evidence that both the intracellular precursor, psCLU, and the mature secreted, sCLU, isoforms constitute proteolytic substrates of the proteasome and the lysosome. Overall our findings indicate that CLU over-expression following proteasome inhibition, relates to both positive gene transcriptional regulation by HSF-1 and post-translational protein accumulation due to reduced proteasomal and lysosomal degradation. CLU manipulation has important

therapeutic potentials and revealing the proteolytic pathways of CLU opens new directions in manipulating and regulating CLU.

YSF–7
Regulation of stromelysin-3 gene expression by Sp1

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Human stromelysin-3 (MMP-11) is a member of the matrix metalloproteinase family that was first described in fibroblasts surrounding breast neoplastic cells. However it has been already detected in macrophages and several tumorigenic cell lines, such as breast and hepatocarcinoma cells. MMP-11 has been described as a predictive tumor marker in serum, being its presence correlated with a bad prognosis. Unlike majority of the members of the MMP family, stromelysin-3 is processed intracellularly and then released in its active form. Although MMP-11 seems unable to degrade components of the extracellular matrix, several substrates have been identified, such as the alpha1-proteinase inhibitor, the insulin-like growth factor binding protein 1, or the laminin receptor; recently, collagen VI has been identified as a new catalytic target. Moreover, this protein not only differs from the other members of the matrix metalloproteinase family in its proteolitic activity, but also in its activation process and specific regulation. Thus, there are two isoforms (alpha and beta) generated by alternative splicing and promoter usage. We have analyzed the expression of both isoforms in human colon adenocarcinoma cells. Luciferase activity assays using different constructions of the stromelysin-3 promoter, have allowed us to detect the basal promoter of both isoforms, located between $-110/+15$ for the alpha, and between $-59/+8$ for the beta isoforms. Furthermore, we have studied the effect of two histone deacetylase inhibitors, butyrate and Trichostatin A, on *stromelysin-3* gene expression. These agents promote a genetic overexpression of all the promoter constructions, being detected even with the shorter construction corresponding to the basal promoter of both isoforms. A further prediction analysis revealed the presence of several regulatory elements on the basal promoter region previously related with the butyrate response, such as Sp1, MAZ or ZBP89. Electrophoretic mobility shift assays confirmed that Sp1, but not MAZ or ZBP89, binds to the basal promoter for both isoforms. Moreover, both the basal promoter activity and the over-expression induced by butyrate was abrogated by Mithramycin A, an antibiotic that binds to GC boxes, thus blocking the Sp1 interaction with DNA and showing the main role of Sp1 on the *MMP-11* gene regulation. Finally, we have detected that the MAP-kinase ERK pathway is involved in the over-expression of stromelysin-3 induced by butyrate.

YSF–8
A novel JNK scaffold protein involved in stress granule formation

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The c-Jun N-terminal kinase (JNK), also known as stress activating protein kinase (SAPK), is part of a signaling cascade composed of MAP2K and MAP3K module. In recent years it became apparent that efficient signaling is mediated by scaffold proteins that simultaneously associate with various components of the MAPK signaling pathway. Even though the scaffold proteins do not display apparent catalytic activity, their role in signal transmission and regulation of the MAPK signaling tires is well accepted. Though JNK MAPK pathway activation promotes cellular stress response, a functional relationship between this pathway and stress granules assembly as a consequence of abortive translation initiation, is unknown. Here we describe the identification of JNK binding protein (JBP), as a novel scaffold protein for the JNK signaling pathway. JBP has no sequence homology to any of the JNK scaffold proteins known today. JBP is highly expressed in skeletal muscle, cardiac muscle, thymus and testes. JBP over-expression in 293T cells results in JNK activation in a dose dependent manner. However, this activation is not followed by transcription activation through the JNK dependent AP-1 transcription factor. Immuno-fluorescence analysis shows that arsenite oxidative stress results in recruitment of JBP to stress granules and phospho-JNK to processing bodies. Furthermore, over-expression of proteins that induce stress granule formation results in localization of JBP and phospho-JNK to the formatted granules. Taken together, these results suggest a role for JBP in translocation of JNK to non-transcriptional compartments of the cell and possible involvement in cellular decisions for mRNA fate following stress.

YSF–9
Can glyoxylate carboligase be a promiscuous enzyme?

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Dipeptidyl peptidase-IV (DPP-IV, CD26, EC 3.4.14.5) is a serine protease that regulates a number of mitogenic peptides involved in cancer development such as neuropeptide Y, stromal cell-derived factor-1alpha, substance P (SP) etc. Our previous results demonstrated that DPP-IV is expressed in glioma cell lines *in vitro* as well as in human gliomas *in vivo*. The aim of this study was to (i) investigate the effects of DPP-IV on growth properties of glioma cells using transfectants with mifepristone-inducible DPP-IV expression, and (ii) elucidate the possible underlying mechanism, in particular modification of the SP pro-oncogenic signaling demonstrated previously in malignant gliomas. SP had, via its cognate receptor NK1, mild growth promoting effect in U373 glioma cells as evidenced by an increase of cells in S phase of the cell cycle. Using the ratiometric indicator Fura-2, we observed that SP triggered calcium signaling in U373 cells. The rise of intracellular calcium was lower in U373 cells overexpressing DPP-IV, but this could not be reversed with a DPP-IV inhibitor. Overexpression of DPP-IV in glioma cells led to substantially decreased growth that could also be observed in another glioma cell line T98G with very low intrinsic expression of the SP receptor. Flow cytometric analysis revealed a decrease of cells in S phase of the cell cycle and a G2/M cell cycle block, which were not influenced when cells were grown in the presence of a DPP-IV inhibitor Diprotin A. Cells highly expressing DPP-IV also exhibited decreased migration and adhesion. In conclusion, using transfected glioma cells with mifepristone-inducible DPP-IV expression, we demonstrate that DPP-IV impairs the growth of glioma cells and may alter intracellular signaling cascades triggered by SP in U373 cells. Moreover, our data suggest that the anti-oncogenic effect of DPP-IV in glioma cells may be independent of its enzymatic activity.

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YSF–10
Drugs of new generation on the base of bis-quaternary salts of 1,4-diazabicyclo[2.2.2] octane

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Development of artificial ribonucleases, compounds able to cleave RNA phosphodiester bonds sequence-selectively, has been the subject of numerous studies during the past decade. These catalysts may be useful as chemotherapeutic agents and for investigation the structures of RNA and RNA-protein complexes in solution. The main aim of our studies is the design and the synthesis of artificial ribonucleases capable of efficient and specific cleavage of RNA under physiological conditions. We synthesized compounds containing two cationic 1,4-diazabicyclo[2.2.2]octane (DABCO) residues, connected by a rigid benzene ring as core structure, and substituted with hydrophobic fragments of different length and structure. The enhanced affinity of such constructs to RNA is provided by the presence of positively charged fragments – a bisquaternary salt of DABCO. The several compounds display high ribonuclease activity. These compounds can inactivate influenza virus A/WSN33/H1N1 *in vitro*, in the cell culture medium and *in vivo*. It has been shown that investigated compounds effectively suppress the flu virus replication. Virus particles treated with artificial ribonuclease effectively protect immunized mice from contamination by fatal dose virus. Hence on the base of such compounds drugs of new generation for precautions and cure of diseases that caused by RNA-content virus can be found. Also these compounds can serve as tool for receipting antivirus vaccine.

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YSF–11
Implication of the inorganic pyrophosphate transporter ank in articular chondrocyte phenotype maintenance

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Background: Articular chondrocyte phenotype is characterized by an expression pattern of genes coding for the extracellular matrix, especially type II collagen. Several wnt genes were described to play a major role in the chondrocyte dedifferentiation process mediated by interleukin-1β (IL-1β) in osteoarthritis. Inorganic pyrophosphate (PPi) was shown to influence osteoarticular cells phenotype. Moreover, we previously demonstrated that ANK was mainly responsible for extracellular PPi (ePPi) generation.
Objectives: We studied the role of ANK and ePPi in the maintenance of articular chondrocyte phenotype, known to be dedifferentiated in osteoarthritis, and the possible implication of Wnt signaling in this process.
Methods: We characterized the dedifferentiation induced in IL-1β-stimulated chondrocytes, and studied the impact of ANK overexpression on this process. We also explored the role of ANK on chondrocyte phenotype using siRNA. Genes expression was assessed by quantitative PCR, and protein expression by immunocytochemistry. Moreover, we studied the Wnt signaling pathways (canonical and non-canonical) involved in these dedifferentiation processes. Finally, the effect of exogenous PPi in culture medium was analyzed on cells subjected to dedifferentiation processes.
Results: IL-1β and transient Ank knock-down induced Wnt-5a mRNA and protein expression, while strongly reducing type II collagen expression, suggesting chondrocyte dedifferentiation. ANK overexpression contrasted the dedifferenciting effects of IL-1β. The canonical Wnt pathway alone (strong expression of β-catenin in the nucleus) was implicated in the dedifferentiation processes. Addition of PPi contrasted both IL-1β and Ank siRNA-induced dedifferentiation processes.
Conclusion: ANK and ePPi are implicated in articular chondrocyte phenotype maintenance, markedly resulting from suppression of Wnt canonical pathway activation. This could open new therapeutic insights in the field of osteoarthritis.

YSF–12
Do retinal rod outer segment disks carry out oxidative phosphorylation?

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Background: Visual transduction in vertebrate retinal rod Outer Segments (OS), compartment devoid of mitochondria, is an energy demanding process. It is currently believed that ATP supply for phototransduction in OS comes from glycolysis, or diffusion from the mitochondria of the rod Inner Segments (IS), but location and timing of both these processes do not seem adequate to provide enough energy for phototransduction. In our recent proteomic analysis of purified bovine rod disks, proteins involved in vision, as well as mitochondria-specific proteins not known to be part of the disk (respiratory chain complexes I to IV and F_1F_o-ATP synthase), were identified. In particular F_1F_o-ATP synthase was catalytically active in disks. Moreover rod OS, even though devoid of mitochondria, selectively stained with mitochondrial vital dyes.
Objectives: The goal of our study is to test the hypothesis that ATP is generated in disks through oxidative phosphorylation by a recruitment of mitochondrial proteins, but not mitochondria.
Methods: Osmotically intact disks were isolated from bovine retinal rod outer segments. Biochemical assays, oxymetry, Rhodamine 123 fluorescence quenching measurements, and imaging techniques (confocal laser scanning and electron microscopy) were employed.
Results: We report a consistent ATP synthesis by purified disks that was inhibited by mitochondrial ATP synthase inhibitors (Oligomycin, Nigericin, DCCD, Antimycin A). This suggests that disk ATP synthase employs a transmembrane electrochemical proton potential difference to synthesize ATP. The presence of a proton gradient across disks is also demonstrated by fluorescence quenching experiments of Rhodamine 123(RH 123). Confocal microscopy of bovine retinas *ex vivo* showed that RH 123 stains OS; rhodopsin and MitoTracker fluorescence co-localize on rod OS. Disks are stained by MitoTracker. The four respiratory chain complexes display an activity comparable to that of mitochondria and are sensitive to the common inhibitors, such as Antimycin A, Rotenone or KCN. Moreover, intact disks consume oxygen when

are energized with NADH or succinate, at a rate similar to that of mitochondria.

Conclusions: Data are suggestive of the presence of an aerobic metabolism in rod disks, that can provide sufficient energy for rod visual transduction. Our findings shed light on many retinal pathologies related to oxidative stress and energy supply in rod OS.

YSF–13
Proteomic analysis to unravel the function of human ribosomal protein S19

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Diamond-Blackfan Anemia (DBA) is a congenital aplastic anemia that selectively involves the erythroid compartment. It has been established that 25% of DBA patients bear a mutated allele of gene encoding the ribosomal protein S19 (Rps19). The finding that RPS19 mutations suppress the expression of the allele has suggested that haploinsufficiency is the main cause of abnormal erythropoiesis in DBA patients. However, some patients carry missense mutations in the RPS19 gene. Rps19 translocates from cytoplasm to the nucleus where it participates to ribosome biogenesis. Recent data suggested that DBA is due to a general defect of protein synthesis in a highly proliferating tissue (1). Using a functional proteomic approach we have recently defined that Rps19 interacts with multiple proteins involved in the ribosome biogenesis and function: NTPases, hydrolases/helicases, isomerases, kinases, splicing factors, structural costituents of ribosome, transcription factors, transferases, transporters, DNA/RNA-binding protein species, dehydrogenase, ligase, peptidase receptor protein, translation elongation factor (2). Coversely, comparative proteomics tools were used to look at the proteins involved in Rps19 mediated pathways. A DIGE (Differential Gel Electrophoresis) experiment was performed on cellular lysates from human eritroleukemia cell line TF-1 in which expression of RPS19 was reduced using siRNA against RPS19 mRNA and from control TF-1 cell line. Differentially regulated proteins were identified by mass spectrometry tools and analysed using Ingenuity Pathway software.

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YSF–14
The role of Mdj1 protein in mitochondrial DNA maintenance

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Background: In eukaryotic cells mitochondria are the main source of ATP, which synthesis engages both nuclear and mitochondrial DNA (mtDNA) encoded proteins. This fact makes maintenance, propagation and distribution of mtDNA important for organism functioning. Numerous human diseases are an effect of mutations along mitochondrial or nuclear genes relevant to mtDNA metabolism. Gene encoding Hsp40 (Dnaja3) can serve as an example. Its deletion in mice inhibits embryonic phase of growth. Tissue specific deletion of Hsp40 gene in cardiomyocytes leads to destabilization of mtDNA and cardiomyopathy as a consequence. In this project we use bakers yeast *Saccharomyces cerevisiae* as the model organism to examine the role of mtHsp40 (Mdj1), Dnaja3 ortholog, in mtDNA maintenance. Mdj1 is a mitochondrial J protein which cooperates with mitochondrial Hsp70 – Ssc1 in folding, reactivation and remodeling of proteins and protein complexes. In that vein, Mdj1 is needed for the folding of mitochondrial DNA polymerase at the top of the temperature range for *S. cerevisiae* growth, 37°C (Duchniewicz et al 1999). Mdj1 is also involved in mtDNA maintenance, even at the optimal growth temperature of 30°C, as deletion of Mdj1 results in loss of respiratory function caused by depletion of functional mtDNA (Rowley et al 1994). Experiments done in our laboratory show that Mdj1 is associated with the mitochondrial nucleoid *in vivo*.

Objectives: We wanted to answer a question: which domains of Mdj1 protein are responsible for mtDNA maintenance?

Methods: Mdj1 mutants lacking its specific biochemical activities, were constructed. The loss of respiration ability *in vivo* was examined using doxycycline repression system of MD1 which allows a precise regulation of protein concentration in the cell by addition or in absence of doxycycline. This method enables us to use plasmid copies of mutated Mdj1 variants in strain *mdj1* containing plasmid encoded *mdj1* gene under tetracycline repression system.

Results: The results of our experiments show that Mdj1 activity in mtDNA maintenance requires interaction with Hsp70, as a single amino acid alteration in the conserved HPD motif in the J-domain results in the same phenotypic effect as the complete absence of the protein. However, J domain alone is not sufficient for mtDNA stability and some C-terminal region of J domain is also necessary.

Conclusion: Functional J domain of Mdj1 is important, but not sufficient for mtDNA maintenance.

YSF–15
Proteomic analysis of parathyroid glands as potential tool to identified cancer biomarkers

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Background: In the last years a growing interest has arisen in the application of proteomic approach to discover biomarkers in many types of cancer but at this time not even one proteomic study has been performed regard to the search of biomarkers in parathyroid diseases. Particularly, parathyroid carcinoma is a rare cause of parathyroid hormone dependent hypercalcaemia (PTHP) with incidence value in PTHP patients less than 1% of cases. However, it is often impossible to distinguish between benign and malignant disease without clear evidence that the tumor is invasive. Local or distant metastases firmly establish the diagnosis of parathyroid malignancy but, at this stage, cure is impossible. Until now, no protein or genetic markers that reliably distinguish adenoma have been identified.

Objectives: In this study, proteomic analysis has been performed to obtain the parathyroid tissue protein map of adenoma.

Methods: All patients included in this study have been submitted to a surgical procedure to remove hyperplastic gland. 16

patients were enrolled in the study and were classified in three groups depending on their calcaemia levels: control group; high iCa^{2+} (A); medium iCa^{2+} (B); low iCa^{2+} (C). The specimens of parathyroid glands were frozen at –80°C immediately after the surgery. Aliquots of samples were homogenized, centrifuged and the pellet was solubilized in rehydration solution. The insoluble material was centrifuged and the supernatant was subjected to two-dimensional electrophoresis (2DE), stained with silver and images were analyzed with Image-Master 2D Platinum software. Spots of interest were identified by mass spectrometry.

Results: About 1150 spots have been detected and comparison of each group of pathological samples with respect to controls allowed us to select 15, 25 and 22 proteins which were differentially expressed respectively in the A, B and C group. Between quantitative differences there is an interesting up-regulation of mitogen-activated protein kinase in each group that is in accordance with the calcaemia increase. Besides to quantitative also some qualitative differences were found in the three groups; for instance we found the peculiar expression of tropomyosin alpha-4 chain in the B group and of aconitase hydratase in the C group.

Conclusion: Our preliminary results demonstrate that proteomic analysis of parathyroid tissue may be the basis for the discovery of potential biomarkers implicated in parathyroid cancer progression.

YSF–16
Dynamics of ERK2 in individual living human cells

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Cells respond to external signals by means of signal transduction cascades. Signaling culminates in translocation of regulatory proteins to the nucleus where they control gene expression. Most studies of these systems are performed on cell averages, masking variability between individual cells. Such signaling systems need to function in the face of large cell-to-cell variation in protein concentrations or cell size. Here we ask how is the response of signaling systems affected by these cell-to-cell variations? Are there aspects of the response which are more robust to cell-to-cell variations than other aspects? We studied the dynamical response of ERK2, a classically studied MAPK signaling protein, by means of fluorescent tagging at the endogenous chromosomal locus and under native regulation in individual living human cells. We monitored the ERK2 nuclear accumulation by time-lapse microscopy upon cell stimulation with specific growth factor. We find that cells show wide basal variation in ERK2 nuclear localization. After signaling, cells show a fold-change response, where nuclear accumulation is proportional to each cells basal level. Nuclear levels then decline and show exact adaptation to the original basal level of each cell. The timing of the ERK2 response is more precise between cells than the amplitude. We further find that in some cells ERK2 exhibits a second pulse of nuclear entry, smaller than the first. The present work of ERK2 dynamics in individual cells shows that despite large variations in basal levels, the system shows exact adaptation, precise timing and a fold-change response mechanism. Two peaks of ERK2 nuclear entry occur in a fraction of cells. This provides a view of this signaling pathway at the individual cell level and suggests that fold rather than absolute changes in nuclear level characterize the response of this pathway.

YSF–17
Further insights into the assembly of the yeast cytochrome bc1 complex

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Background: The yeast cytochrome bc_1 complex, also known as complex III, is a dimeric respiratory enzyme embedded in the inner mitochondrial membrane. Each monomer is made up of three catalytic subunits containing redox prosthetic groups and seven non-redox subunits with unknown function.

Objectives: The present study focuses on yeast complex III biogenesis, in order to propose a possible pathway of assembly of the functional complex.

Methods: We have created mutant yeast strains in which single or pairs of genes encoding bc_1 subunits had been deleted. The mitochondrial membranes isolated from wild type and mutant strains were then analyzed by two dimensional electrophoresis (BN-PAGE and SDS-PAGE) and immuno-blotting.

Results: In wild type mitochondrial membranes complex III was detected in the free dimeric form and in two super-complexes with one or two copies of the cytochrome *c* oxidase complex. The analysis of mutant mitochondrial membranes revealed the presence of a common set of bc_1 sub-complexes. In particular, we have characterized a bc_1 sub-complex of about 500 kDa, which could represent a stable intermediate during the assembly of complex III, because of its wide distribution in distinct yeast deletion strains and its characteristics of stability.

Conclusion/Application to practice: The characterization of this core structure may help in clarifying the complicated process of bc_1 assembly in eukaryotic mitochondria. Indeed, the extreme importance of this research is due to the fact that lack of assembly of this respiratory complex is the cause of severe human pathologies.

YSF–18
MAFbx/Atrogin-1 controls eIF3-f activity in skeletal muscle atrophy by targeting multiple C-terminal lysines

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Skeletal muscle (SM) mass depends upon a dynamic balance between anabolic and catabolic processes. SM hypertrophy is characterized by an increase of the diameter of muscle fibers and increased protein synthesis, mainly by activation of the IGF1/Akt/mTOR pathway. Muscle loss occurs as the result of a number of disparate conditions including cancer, diabetes, AIDS, sepsis, renal failure, aging, cachexia, and other systemic diseases. These diverse conditions result in reduced protein synthesis and increased protein breakdown. The process of atrophy is characterized by the activation of the ubiquitin-proteasome proteolysis pathway. The E3-ligase MAFbx is upregulated in multiple models of atrophy and appears to be essential for accelerated muscle protein loss. Recently, we showed that MAFbx interacts with the initiation factor eIF3-f for polyubiquitylation and further proteasome-mediated degradation during SM atrophy (1). eIF3-f is a regulatory subunit of the eIF3 complex that interacts directly

with mTOR and S6K1 to coordinate the assembly of the preinitiation complex. Furthermore, overexpression of eIF3-f in SM induces a marked hypertrophy associated with an increase of sarcomeric proteins (2). Thus, the specific targeting of eIF3-f by MAFbx may account for the decreased protein synthesis observed in multiple types of SM atrophy. In the present work, we have mapped the region of eIF3-f responsible for its proteolysis. We showed that six lysines located in the C-terminal domain are required for fully MAFbx- mediated polyubiquitylation and degradation by the proteasome. In addition, site-directed mutagenesis of these six lysines (mutant $K_{5-10}R$) displayed hypertrophic activities *in cellulo* and *in vivo,* characterized by an increase of mean myotubes diameters and the cross sectional area of muscle fibers, accompanied with a higher phosphorylation of S6K-1, 4E-BP1 and the rpS6. Furthermore, this hyperactive mutant was able to protect against starvation-induced SM atrophy (3). Taken together, our data demonstrate that the C-terminal modifications, believed to be critical for proper eIF3-f regulation, are essential and contribute to a fine-tuning mechanism that plays an important role for eIF3-f function in SM.

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YSF–19
Role of ETS1 in paclitaxel and vincristine resistance development in MCF-7 cells

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Background: ETS1 proto-oncoprotein is a member of the ETS family of transcription factors that share a unique DNA binding domain, the ETS domain. ETS1 and mutant p53 interaction is one of the major mechanisms to upregulate Multiple Drug Resistance 1 gene (*MDR1*) expression at transcriptional level. Among the genes that respond to ETS1 are those that code for matrix metalloproteases MMP-1, MMP-3, MMP-9.

Objectives: In the present study the aim was to assess the involvement of *ETS1* and the genes which encode the proteins interacting with ETS1 in drug resistance in MCF-7 breast cancer cells.

Methods: Drug resistant sublines (MCF-7/400 nMPac and MCF-7/120 nMVinc) were developed from sensitive MCF-7 cells (MCF-7/S) by paclitaxel and vincristine applications in dose increments. Degree of resistance was evaluated by XTT assay. RNA was isolated from sensitive and resistant MCF-7 cells and cDNA microarray analysis was performed using Affymetrix Gene Chip (Human Genome U133 Plus 2.0 Array) in duplicate experiments. GeneSpring GX 7.3.1 Software was used for data analysis. Gene expressions that significantly changed 2-folds or more in resistant sublines compared to sensitive cells were selected. Microarray data was supported by immunocytochemistry for the most well known drug resistance protein P-gp which is encoded by *MDR1* gene.

Results: MCF-7/400 nMPac and MCF-7/120 nMVinc cells were resistant to selective drugs 150- and 30-fold respectively. According to microarray data *ETS1* and *MDR1* genes were highly overexpressed in MCF-7/400 nMPac and MCF-7/120 nMVinc. Matrix metalloproteinase-1gene (*MMP-1*) was also tremendously upregulated in MCF-7/120 nMVinc cells. Immunocytochemistry results confirmed the microarray results such that P-gp was highly overexpressed in resistant sublines compared to sensitive MCF-7 cells.

Conclusions: High *ETS1* expression levels in vincristine and paclitaxel resistant sublines may have implications on the upregulation of the transcription of *MDR1* gene. Overexpression of *ETS1* gene in resistant cells may have contributed the drug resistance of these cells. Furthermore, the upregulation of *MMP1* and *MMP9* in MCF-7/120 nMVinc may contribute the invasive characteristics. However this interaction was not that clear for paclitaxel resistant subline. The findings indicate a relationship between *ETS1* overexpression and drug resistance development in breast cancer cell line.

YSF–20
Effects of clusterin knock down on prostate cancer progression in the TRAMP model

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The TRAMP transgenic mouse model spontaneously develops prostate cancer (CaP) because of specific expression of the SV40 antigen transgene in the prostate tissue, displaying progression from prostatic intraepithelial neoplasia (PIN) by 8–12 weeks of age to adenocarcinoma and distant metastases by 24–30 weeks of age and mimicking human disease. Clusterin (Clu) gene is highly conserved in animal tissues. Its protein products have been found implicated in modulating cell survival and neoplastic transformation. At present, its role in human cancer progression is highly controversial. Changes in Clu expression have been documented in different malignancies. We found that Clu is down regulated during CaP progression in humans and in the TRAMP model. We reasoned that knock down of Clu in a suitable CaP model would affect disease progression. To properly address this, we generated TRAMP-$Clu^{+/-}$ and TRAMP-$Clu^{-/-}$ mice by crossing genetically compatible TRAMP with Clu KO mice. Preliminary data in Clu KO mice showed that, surprisingly, PIN was present in five out of eight $Clu^{+/-}$ and six out of nine $Clu^{-/-}$ at 40 weeks of age. Differentiated CaP was also found in two out of eight $Clu^{+/-}$ and three out of nine $Clu^{-/-}$ mice. Wild type siblings did not show any cancer lesions. Higher Ki-67 labeling index was found by IHC assays in the prostate tissue of $Clu^{+/-}$ and $Clu^{-/-}$ mice if compared with wild-type controls. In addition, we found higher *P*65 NF-kB staining in the prostate of CLU deleted mice compared to wild type controls. After crossing Clu KO with TRAMP mice, we found that survival at 28 weeks of age was 100% for TRAMP $Clu^{+/+}$, but 83% and 70% for TRAMP $Clu^{+/-}$ and TRAMP $Clu^{-/-}$, respectively. Furthermore, while all TRAMP $Clu^{+/+}$ mice examined at necropsy developed large *in situ* prostate tumors and rare metastatic diffusion to lymph nodes, all TRAMP $Clu^{+/-}$ and TRAMP $Clu^{-/-}$ mice showed cancer invasion spreading in many different body sites including kidney and liver. In situ neoplastic and metastatic lesions, all positive for SV40 antigen transgene, were also consistently Ki67- and NF-kB-positive in TRAMP $Clu^{-/-}$ if compared to TRAMP $Clu^{+/+}$ mice. Finally, 8% of TRAMP $Clu^{-/-}$ females, normally cancer free, were affected by tumours, mostly localized to thyroid and uterus but also to lymph nodes and lungs. In conclusion, our data indicate that suppression of Clu in the TRAMP model induces a more invasive disease because deletion of CLU leads to

activation of NF-kB, a potentially oncogenic transcription factor important for proliferation and survival of prostate cells.

YSF–21
Effects of BRCA1-BRCT mutations in structure, function and cellular localization of BRCA1 protein

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The product of the brca1 gene is central to recombination reactions and thus contributes significantly to maintain the integrity of the genome. BRCA1 protein is strongly involved in DNA repair and cell cycle control through interactions with other partner proteins like BACH1, CtIP, p53 and Rad51. Mutations at the two C-terminal tandem (BRCT) repeats of BRCA1, disrupting BRCA1 interactions with other proteins, were identified in breast tumor patients. Biophysical analysis on the secondary structure, the thermodynamic stability and the modification in binding capacity of the recombinant and purified wt and mutated BRCT domains with BACH1 and CtIP have already been studied *in vitro*, by employing Circular Dichroism Spectroscopy (CD), Differential Scanning Microcalorimetry (DSC) and Isothermal Titration Calorimetry (ITC). Our currently available biophysical experiments clearly demonstrated that certain pathogenic mutations (V1696L, M1652I, M1775K, M1783T, V1809F, P1812A) of the BRCA1-BRCT cause changes in the stability of the domain and influence the binding affinities with synthetic phosphopeptides, corresponding to BACH1 and CtIP binding sites. In order to investigate the effects of these pathogenic mutations of BRCA1-BRCT in protein's localization fused GFP-BRCA1 mutants demonstrated and expressed in MCF7 and HeLa cells. Analysis of the intracellular localization, as well as the co-localization-interactions with p53 and Rad51, were performed with fluorescence microscopy, confocal microscopy and immunoprecipitation. The current preliminary experiments indicate that pathogenic mutations of BRCT domain with structurally unstable feature *in vitro*, affect the intracellular localization of the BRCA1 protein and its 'cross-talk' with other protein partners. The anticipated results will support the elucidation of the effect of various pathogenic BRCT mutations on the missfunction of BRCA1 nuclear DNA repair replication and cell cycle control at the protein level.

YSF–22
Effect of inhaled corticosteroids on lymphocyte MDR1 gene expression and clinical relevance of MDR1 polymorphism in asthmatic children

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Asthma bronchiale is a syndrome characterized by airway obstruction, varying both spontaneously and as a result of therapy. Majority of asthma bronchiale patients require daily anti-inflammatory treatment to maintain appropriate symptom control and life quality. But the successful pharmacotherapy of various diseases, including autoimmune diseases, is often limited by multidrug resistance. In the response to the corticosteroid therapy are discussed various factors like functionality of glucocorticoid receptors, impaired pathways and tissue glucocorticosteroids accessibility. In recent years it has been demonstrated that alterations in the expression and activity of the MDR transporters are seen in numerous tissues during an inflammatory response. The objective of our study was to verify the possible associations between the effects of inhaled corticosteroids and montelukast (antagonist of cysteinyl receptors) and the level of MDR1 (PGP) expression in the lymphocytes of asthmatic patients. In parallel, the MDR1 gene polymorphism was also studied. In the group of 102 the children we observed significant decrease of PGP expression on peripheral blood lymphocytes of children treated with corticoids compared to those without corticoid medication ($p < 10^{-4}$). We also found differences in frequency of the MDR1 genotype for SNP C3435T (rs1045642) and its association with expression of the MDR1 protein ($p < 10^{-3}$). These data suggest that the gene expression and/or MDR1 variants may have clinical relevance in asthmatic patients modulating bioavailability and/or the anti-inflammatory effects of local corticosteroids.

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YSF–23
New insights into the role of insulators in *D. melanogaster*

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Background: Insulators are thought to divide eukaryotic genomes into independent transcriptional units, protecting promoters from inappropriate activation by enhancers from neighboring genes in position-dependent manner. Although much of the research was made to understand the main role of insulators in the regulation of gene expression, it's still poorly known. Here we investigated the role of two insulators that are placed endogenously downstream the *yellow* and *white* genes.
Methods: Plasmid construction, the phenotypic scoring assay, germ line transformation, genetic crosses, X-ChIP.
Results: With the use of transgenic model system in *D. melanogaster* we show that 1A2 and Wari insulators are able to directly interact with promoters of *yellow* and *white* genes, respectively. Moreover, deletion of the Wari insulator downstream the *white* gene in transgenic constructs leads to significant decrease of the *white* gene expression that argues the important role of at least some insulators in gene transcription.
Conclusion: The results provide new insights into the role of insulators in the control of gene expression.

YSF–24
Alternative splicing of Metal-responsive Transcription Factor (MTF-1)

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Metal responsive control of gene expression allows organisms to adjust the concentration of essential metal ions such as Zn^{2+} and Cu^{2+}, within an acceptable range and cope with detoxification of heavy metals (Cd^{2+}, Pb^{2+} and As^{3+}) with no biological function. Metallothioneins (MTs) are widely inducible at transcriptional level by a variety of metals and other stress conditions such as accumulation of reactive oxygen species, hormones, cytokines. Transactivation of metallothionein genes involves the

Metal-responsive Transcription Factor (MTF-1) a metal responsive element (MRE) binding, zinc sensitive protein. In this study we present the first evidence for an *mtf-1* splicing variant (*mtf-1.1a*), originated from the brain of unstressed common carp. We have follow the level of *mtf-1.1a* mRNA in different tissues of unstressed animals and the effect of heavy metal loading (Cd and As) on the alternative splicing of *mtf-1.1* transcript. The splice variant of *mtf-1.1* mRNA codes for a truncated MTF-1.1 protein. The lack of a 103 nucleotides internally in the *mtf-1.1a* transcript, between positions 1047–1149, results in a frame shift causing an early termination of translation. The putative MTF-1.1a protein consists of the first 349 amino acids of MTF-1.1 followed by an additional 64 amino acids, which dońt resemble at all to the corresponding region of MTF-1.1. The 349 amino acid covers the six Zn-finger DNA binding domains, the nuclear localization (NLS) and the nuclear exporting (NES) signals and the first 12 amino acid of the acidic region. Under unstressed conditions *mtf-1.1a* was detected in all tissues examined, but the liver, with the highest level in the brain. Arsenic alters the level of both *mtf-1.1* and *mtf-1.1a* transcripts in an isoform and tissue-specific manner. Cadmium had no measurable effect on the alternative splicing of *mtf-1.1* in the liver, while the amount of both *mtf-1.1* transcripts gradually decreased in the brain.

YSF–25
Protein engineering and electrode modification for the creation of a P450 based biosensor

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Background: P450 BMP is the haem domain of cytochrome P450 BM3 from *Bacillus megaterium*.
Objectives: In this work P450 BMP (wild type and mutants) has been characterized with spectroscopic and electrochemical experiments to better understand the factors that influence the response of the protein on the electrode surface when creating a biosensor. We present data to examine its ability to covalently bind to electrodes and to hydroxylate organic substrates while immobilized.
Methods: Site-directed mutagenesis and functionalization of gold surfaces have been combined to obtain a stable immobilization of BMP. Tapping mode atomic force microscopy (TMAFM) was exploited to understand whether the protein was immobilized on the surface. Electrochemistry experiments were performed to test the ability of the protein to exchange electrons with the electrode surface and also to electrochemically catalyse the turnover of the non natural substrate naphthalene.
Results: TMAFM experiments carried out on the first spacer derivatized gold led to good images with expected molecular heights for the wild type and the C156S mutant. These samples also gave measurable electrochemical signals with midpoint potentials of −48 and −58 mV for wild type and C156S respectively. The second spacer led to variability on the molecular heights and the electrochemical response. TMAFM also shows that the double mutant and the C62S did not lead to stably immobilized BMP, confirming the necessity of the solvent exposed C62 for the linkage.
Conclusion/Application to practice: The naphthalene assay would prove the ability of P450 BMP to work as a biosensor when immobilized.

YSF–26
Using the nematode enzyme as template for elucidating regulatory mechanism of phenylalanine hydroxylase

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Introduction: Phenylalanine hydroxylase (PAH) is a tetrameric enzyme which in mammals is mainly present in the liver. Its strict regulation is very important in order to maintain the appropriate level of phenylalanine to (i) avoid the disease phenylketonuria (PKU) characterised by mental retardation and (ii) provide a continuous supply of tyrosine for protein synthesis. We have previously characterised a monomeric bacterial PAH and, in this work, the tetrameric PAH from the nematode *Caenorhabditis elegans* (cePAH). Previous work in our lab has indicated that the main function of cePAH is in melanogenesis and that this enzyme lacks the sophisticated regulatory mechanisms found in the mammalian enzyme. In order to identify residues important for regulation in the mammalian enzyme, ''humanised'' versions of cePAH have been created and studied.
Materials and Methods: Mutant forms of cePAH with mammalian residues in selected regions (QN215KY, N415D, D236T, QN215KY/N415D and QN215KY/D236T) were prepared by site-directed mutagenesis. Activity measurements as well as Isothermal titration Calorimetry and Differential Scanning Calorimetry have been used to characterise the activity, stability and stoichiometry of phenylalanine-binding of these mutants in comparison with wild-type human PAH and cePAH.
Results: The mutant cePAH are approaching the values obtained for the human enzyme with regards to positive cooperative response and binding stoichiometry.
Conclusions: Using cePAH as a template, this work has identified several amino acid residues important for regulating the mammalian enzyme. It has also provided insights into the basis for the evolution of sophisticated regulatory mechanisms in an important metabolic enzyme. It appears that the importance of proper regulation and prompt response of the enzyme to elevated substrate level have evolved with the complexity of the organism.

YSF–27
Membrane interactions of HIV fusion inhibitors studied using a biophysical approach

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The HIV fusion process mediated by the gp120-gp41 complex occurs in an extreme confinement between the viral envelope and the cellular citoplasmatic membrane. The efficacy of a HIV fusion inhibitor may, therefore, be related to its ability to interact with membranes. We studied the interaction of the HIV fusion inhibitor sifuvirtide, a 36 aa negatively charged peptide, with lipid vesicles. Since this peptide has aromatic residues, Fluorescence Spectroscopy techniques (both steady-state and time-resolved) were mainly used. Results showed no significant interaction with both zwitterionic fluid phase and cholesterol-enriched membranes; however significant partition to fluid phase cationic membranes were observed. Similar results were obtained using a quenching approach with acrylamide. In the DPPC gel phase, however, an adsorption at the surface of these membranes was detected by using a differential quenching approach with lipophilic probes, as well as by Förster Resonance Energy Transfer.

Our results show a selectivity and specificity of the peptide toward rigid domains, where most of the receptors are found, and help explain the importance of the interaction with membranes in the improved efficacy of sifuvirtide compared to other fusion inhibitors (T20 and T1249), by providing a local increased concentration of the peptide near the fusion site on both cellular and viral membranes [1]. As the HIV viral membrane is enriched on DPPC (and other saturated phosphatidylcholines) relative to the host membrane [2], a specific interaction of sifuvirtide, related to its mode of action, can also be hypothesized. Furthermore, since a Ca^{2+}-binding site was described to be present in gp41 CHR domain [3]; we tested the effect of Ca^{2+} in the interaction of sifuvirtide with membranes. The electrostatic interactions with cationic vesicles were also studied in more detail. Beside fluorescence, Atomic Force Microscopy (AFM) was used to study the interaction of sifuvirtide, but also T20 and T1249, with supported lipid bilayers.

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YSF–28
MicroRNAs are essential for development and function of inner ear hair cells in vertebrates

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Background: The mammalian inner ear contains the cochlea and the vestibule that are responsible for hearing and balance, respectively, while the fish inner ear resembles the mammal vestibule. Hearing loss and vestibular dysfunction often involve inner ear developmental defects or degeneration of the inner ear sensory hair cells (HCs). MicroRNAs (miRNAs) are 17–25 nt double-strand RNAs that inhibit the translation of target mRNAs and affect, directly or indirectly, the expression of a large portion of the protein-coding genes. The ribonuclease Dicer is required for the production of mature and functional miRNAs.

Objectives: Our goal is to study the expression and roles of miRNAs in the vertebrate inner ear.

Methods: Using the *cre-loxP* recombination system, a mutant mouse in which *Dicer1* is knocked-out conditionally in inner ear HCs, where *Pou4f3* promoter is expressed, was created. miRNAs from wild type mouse inner ears were profiled using microarrays. Real time qRT-PCR and *in situ* hybridization were used to study spatial and temporal expression patterns of selected miRNAs. Morpholinos were used to knock-down isolated miRNAs in zebrafish embryos. To suggest putative target mRNAs whose translation may be inhibited by selected miRNAs, we combined bioinformatics-based predictions and mRNA expression data. The dual luciferase assay was used to confirm some putative targets.

Results: The mutant mouse demonstrated that miRNAs are crucial for postnatal survival of functional HCs of the inner ear. We identified miRNAs that have a role in the developing inner ear, by combining miRNA transcriptome analysis, spatial and temporal expression patterns, and bioinformatics. Microarrays revealed similar miRNA profiles in newborn mouse whole cochleae and vestibules, but different temporal and spatial expression patterns of six miRNAs (miR-15a, miR-18a, miR-30b, miR-99a, miR-182 and miR-199a) may reflect their different roles. A subset of these miRNAs was also shown to be crucial for zebrafish inner ear development and morphogenesis. Putative target mRNAs that are expressed in the inner ear sensory epithelia were identified for several miRNAs that are also expressed in these tissues. Indirect evidence supports our hypothesis that Slc12a2, Cldn12 and Bdnf mRNAs may be targets for miR-15a.

Conclusion: Our data support the hypothesis that inner ear tissue differentiation and maintenance are regulated and controlled by conserved sets of cell-specific miRNAs in both mouse and zebrafish.

YSF–29
Regulation of uncoupling protein 2 by silybin and its derivatives in rat neonatal cardiomyocytes

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Uncoupling protein 2 (UCP2) is a carrier protein located in the inner mitochondrial membrane, allowing mild uncoupling of mitochondrial respiration. In rat neonatal cardiomyocytes, overexpression of UCP2 confers tolerance to oxidative stress via diminished mitochondrial Ca^{2+} overload and reduced generation of ROS. Thyroid hormones are major regulators of cellular respiration and UCP2 levels are up-regulated by thyroid hormones. Hyperthyroidism and hypothyroidism are associated with increased and decreased mitochondrial respiration rates, respectively. Silibinin, also known as silybin, is the major active constituent of silymarin, the mixture of flavonolignans extracted from seeds of milk thistle (*Silybum marianum*). It is used in the treatment and prevention of liver diseases because of its hepatoprotective (antihepatotoxic) properties. We used silybin and its derivates, 2,3-dehydrosilybin and 7,20-di-*O*-methylsilybin, to evaluate their effect on UCP2 expression, which we determined using western blot and real-time PCR. We demonstrate that L-thyroxine (8 ng/ml) induces UCP2 protein expression in rat neonatal cardiomyocytes after 24 h of treatment. Expression of UCP2 was restored to its original level when cardiomyocytes were pre-incubated for 30 min with increasing concentrations of silybin and its derivatives. We also studied LDH and MTT activities, and membrane potential (JC-1) in cardiomyocytes to assess toxicity of the substances. Interestingly, 2,3-dehydrosilybin caused decrease in membrane potential while protecting cardiomyocytes against H_2O_2 exposure. We hypothesize that silybin and its derivatives modulate UCP2 expression via affecting thyroid receptor transcriptional activity.

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YSF–30
Novel inhibitors of cytokinin oxidase/dehydrogenase and their potencial use for *in vivo* studies

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Cytokinins are plant hormones controlling numerous processes associated with plant growth and development. Cytokinin

oxidase/dehydrogenase (CKX, EC 1.5.99.12) is enzyme involved in their irreversible degradation and thus regulates levels of endogenous cytokinins in plants. Lower CKX expression was shown to be reason of increased grain number in an *indica* rice variety indicating that modulation of cytokinin catabolism may lead to improvement of traits of important crops. Recently we have described synthesis of substituted 6-anilinopurines as a new group of potent CKX inhibitors (1). We studied their biological activity in classical cytokinin bioassays as well as in the receptor and CKX degradation assay. Compared to thidiazuron, a synthetic cytokinin with strong biological activity resulting from strong activation of cytokinin receptors and CKX inhibition, most of the 6-anilinopurines inhibited CKX much more strongly, but their sensing by cytokinin receptors was much weaker. Here we present biological characterization of one of these compounds designated MZ02Cl. We show that MZ02Cl competitively inhibits activity of recombinant *Arabidopsis* CKX enzymes *in vitro* in both oxidase and dehydrogenase modes in dose-dependent manner. Co-crystallization of MZ02Cl with ZmCKX1 confirmed the binding of the compound into the enzyme active site. MZ02Cl inhibited the degradation of exogenously applied radiolabelled isopentenyladenosine in intact *Arabidopsis* seedlings. *In vivo* function of the inhibitor was further confirmed by treatment of transgenic tobacco and *Arabidopsis* plants overproducing AtCKX1 and AtCKX2. MZ02Cl application led to the release of the shoots of the treated plants from growth inhibition and complementation of wild-type phenotype. Treatment of wild-type plants resulted in altered shoot and inflorescence development, i.e. shortening of internodes length, increase of flower size and number, and consequent higher fruit yield. Our results indicate that MZ02Cl modulates endogenous cytokinin level *in planta* and may have find interesting applications in studies of cytokinin action as well as a growth regulator for modifying the traits of crop plants.

Reference:

1. Zatloukal *et al., Bioorg Med Chem.* 2008; **16**: 9268–75.

YSF–31
Interaction between mucin and arabinogalactan, a no-viscous polymer promising for the treatment of dry-eye

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Background: Dry eye syndrome is associated with tear film deficiency, as a consequence of either an insufficient supply or an excessive loss, and with anomalous tear composition. Artificial tears are usually characterized by an high viscosity, which should increase their residence on the ocular surface. However, high viscosity leads to several unpleasant disadvantages. Arabinogalactan (AG) a low-viscosity natural polysaccharidic molecule has been shown to exert a corneal protective action. This study is devoted to assess mucoadhesive properties of AG, by evaluating its ability to interact with mucins.

Methods: AG, pharmaceutical grade, isolated from conifers of the genus *Larix* (Larch) was supplied by Opocrin, S.p.A. Mucin MUC1, from submaxillary glands, was purchased from Sigma-Aldrich. AG content was evaluated spectrophotometrically upon reaction with the anthrone reagent. Both gel filtration chromatography and frontal gel chromatography approaches were adopted to study the interaction between AG and mucin. The first approach is based on the shift possibly occurring in the elution profile of a ligand when subjected to gel filtration chromatography in the presence of the target. The second one is based on the measurement of the ligand bound to the target while emerging from a chromatographic column equilibrated with different ligand concentrations.

Results: Mucin and AG display, when chromatographed separately on a Sephacryl S300 column, well distinct elution peaks. A significant change in the elution profile of AG, compatible with a transient coelution of the two molecular species, is observed when the polysaccharide is chromatographed together with mucin. On the contrary, no effect is exerted on the elution profile of AG by different proteins and glycoproteins with molecular mass comparable with mucin. Frontal gel chromatography experiments were performed using 1 mg/ml mucin and AG concentrations ranging from 0.06 to 0.23 mg/ml. The effectiveness of the interaction process was assessed through the determination of the dissociation constant for the AG:mucin complex.

Conclusions: The ability of AG to interact with mucin is the base to further the mucoadhesive features of this natural polysaccharide to be used as a terapeutic tool to counteract dry eye syndrome.

YSF–32
Intracellular transport of retroviral envelope glycoproteins and their interaction with a structural polyprotein Gag

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The transport of retroviral envelope glycoproteins (Env) and their interaction with a structural polyprotein Gag in a late phase of virus life cycle is poorly understood. We are focused on a D-type retrovirus i.e. Mason-Pfizer monkey virus (MPMV) that assembles its particles within a cytoplasm of an infected cell and these particles are then transported to the plasma membrane. It has been suggested that such transport occurs due to an interaction of preassembled particles especially matrix region of Gag with Env that is exposed on a surface transport vesicles. To address this issue, constructs for the expression of envelope glycoproteins fused with fluorescent protein (blue fluorescent protein, yellow fluorescent protein, cherry fluorescent protein) and constructs for expression of retroviral structural polyprotein Gag fused with green fluorescent protein (GFP) were prepared. Similarly, constructs for the expression of envelope glycoproteins were prepared by inserting the gene encoding fluorescent protein in the end of cytoplasmic tail of transmembrane subunit of this glycoprotein. Constructs for expression of polyprotein Gag fused with GFP were prepared by inserting the gene encoding GFP into expression vector with codon optimized gene for polyprotein Gag and with sequence for envelope glycoproteins. The correct position of inserts was verified by sequencing. To investigate localization of proteins, COS-1 cells (African green monkey kidney cell line) were harvested at various times following the transfection and analyzed by fluorescence microscopy. Expression of the fusion proteins as well as incorporation of the glycoprotein into assembled virus particles were proved by pulse chase experiments. The transport of both polyproteins in real time was analyzed in three cell lines i.e. HeLa, COS-1 and CMMT-1. Movement of viral proteins tagged by fluorescent proteins inside the transfected cells and their potential interactions were observed by Delta Vision Live Imaging Microscope in real time. The initial results suggested that Gag co-migrates with Env by a vesicular transport. However, further investigation is required to verify this conclusion.

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YSF–33
Model of spatial structure of catechol 1,2-dioxygenase from *Pseudomonas putida* N6 strain

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Environmental strain *Pseudomonas putida* N6 belongs to the collection of Department of Biochemistry, Silesian University and exhibits remarkable ability to degrade aromatic compounds. In the crude cell extract of this strain activity of catechol 1,2-dioxygenase was revealed, and the attempts of amplification of appropriate gene target sequence has been made. The aim of this work was to determine the hypothetical spatial structure of catechol 1,2-dioxygenase of *Pseudomonas putida* N6 strain on the basis of nucleotide sequence. Additional goal of this work was to identify ligands of metal ion in the dioxygenase active site. Amplification of catechol 1,2-dioxygeanse gene, construction of vectors and transformation of the laboratory strains were carried out by the standard procedures used in molecular biology. Nucleotide sequence of gene, which was obtained after amplification, exhibited sequence similarity to other 1,2-dioxygenases. DNA transcription and analysis of amino acids sequence were performed using CLC Free Workbench 4.0.1. Start codon as well as stop codon were found in the transcript of nucleotide sequence of catechol 1,2-dioxygenase gene. Obtained amino acids sequence consists of 301 amino acids. Using 3D-JIGSAW Protein Comparative Modelling Server (Cancer Research, UK, http://www.bmm.icnet.uk/~3djigsaw) there was made an attempt to determine the spatial structure of catechol 1,2-dioxygenase of *Pseudomonas putida* N6 strain. Spatial structures as x.*pdb* files were analysed using RasMol 2.6. Analysis of 3D-structure revealed the presence of two domains. N-terminal domain consisted of five α-helixes, and C-terminal domain have a predominantly β-sheet structure. As iron (III) ligands in the dioxgenase active site the following amino acids were typed: His-212, His-210, Tyr-152 and Tyr-186. Isolation and characterisation of environmental strains and construction of new bacterial genotypes able to degrade the wide spectrum of aromatic xenobiotics seem to be indispensable steps in the efficient purification of polluted environment. That's why knowledge of degradative pathways, enzymes and suitable genes of large number of microorganisms is required.

YSF–34
Isolation and biochemical characterization study of lipid rafts from Atlantic cod (*Gadus morhua*) intestinal enterocytes

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Membrane lipid rafts are glycosphingolipid/cholesterol-enriched membrane micro-domains that have been extensively studied during the past two decades. However, to the best of our knowledge, no studies have yet been performed on lipid rafts from the intestinal brush border membrane (BBM) of ray-finned fishes (Actinopterygii). Our aim was to isolate and perform biochemical characterization of lipid rafts from the BBM of Atlantic cod (*Gadus morhua*) intestinal enterocytes to confirm their existence and if they showed similarity to lipid rafts from other species in terms of lipid and protein content. To validate the isolation process, we assayed marker enzymes for sub-cellular organelles, including alkaline phosphatase (AP) and leucine aminopeptidase, both well-known marker enzymes for BBM and lipid rafts. AP was mapped in tissue-slices after by immunostaining by confocal microscopy, enzyme activity, and Western blotting. We also performed lipid analysis on BBM and lipid rafts by thin-layer chromatography and ^{31}P-NMR. Proteomics studies where performed by MALDI (Matrix-assisted laser desorption/ionization) and LC-ESI (Liquid-chromatography electrospray ionization) mass spectrometry from trypsin digested SDS-PAGE samples. All methods showed enrichment of AP in both BBM and lipid rafts fraction, ^{31}P-NMR gave higher content of sphingomyelin then previously reported and lower content phosphatidylcholine in the BBM, but sphingomyelin was highly dominant in the lipid rafts together with cholesterol. Various proteins have been associated with our lipid raft preparation such as aminopeptidase-N, prohibitin, beta-actin, and villin 2. The existence of lipid rafts containing previously reported lipid raft proteins has, therefore, been confirmed here for the first time in a ray-finned fish.

YSF–35
Connexin43 is involoved in the effect of endothelin-1 on astrocyte proliferation

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ET-1 was initially identified as a potent vasoconstrictor peptide produced by vascular endothelial cells and has been found to have multiple and very different biological activities in the central nervous system (CNS). Thus, ET-1 is present in brain endothelial cells, neurons and astrocytes, and its secretion increases in several phatologies, such as astrocytic tumors acting as a mitogenic factor. Astrocytes are known to be interconnected by gap junctions and thereby form a junctional synctium. Gap junctions in astrocytes are mainly composed of the channel protein connexin43 (Cx43), which has been considered a tumor supressor protein. In fact, a decrease or loss of Cx43 expression is usually observed in gliomas, and the expression of Cx43 is inversely correlated with the degree of malignancy. In previous studies, we showed that ET-1 increased astrocyte proliferation. These effects were similar to those observed with other gap junction inhibitors, such as carbenoxolone (CBX). Because treatment with ET-1 or CBX downregulates the expression of Cx43, in this study we adressed the possible role of Cx43 in the mitogenic effects of ET-1. To do so, we investigated the effect of ET-1 on astrocyte proliferation in astrocytes whose Cx43 had been silenced by siRNA and in astrocytes obtained from Cx43 KO mice. Our results showed that the effects of ET-1 on the upregulation of proliferation marker Ki-67, on retinoblastoma phosphorylation on Ser780 and on the upregulation of cyclins D1 and D3 were affected by the levels of Cx43. Furthermore, by itself, silencing Cx43 promoted the upregulation of cyclin D3, the phosphorylation of pRb on Ser 780 and the expression of Ki-67. In conclusion our results indicate that Cx43 participates in the mitogenic effects of ET-1 in astrocytes and that the downregulation of Cx43 is a mitogenic signal for astrocytes. Interestingly, although the rate of growth in Cx43 KO astrocytes has been reported to be reduced, we observed that an acute reduction in Cx43 by siRNA increased proliferation.

YSF–36
Regulation of mitochondrial sulfide oxidation: glutamate protects mammalian cytochrome oxidase from inhibition by H2S

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Background: Hydrogen sulfide is enzymatically produced in mammalian tissues and functions as a gaseous transmitter. Nevertheless, H_2S is also highly toxic as it inhibits mitochondrial respiration at the level of cytochrome oxidase. Interestingly, the pathway catalysing sulfide oxidation transfers electrons into the respiratory chain. Thus, the enzyme that is inhibited by H_2S is also required for its detoxification. A protective mechanism is likely to exist since the physiological sulfide concentrations detected in different tissues are much higher than the inhibitory concentration for isolated mitochondria.
Objectives: The present study aimed to identify cytosolic metabolites that can affect mitochondrial sulfide oxidation.
Results: Mitochondria isolated from rat liver were able to use low concentrations of sulfide (≤ 20 μM) as a respiratory substrate. Glutamate, but neither of its reaction products shifted the threshold for inhibition of cytochrome oxidase towards higher sulfide concentrations. As a result, 50 μM H_2S was rapidly oxidized with constant rates of oxygen consumption and ATP production, which were comparable to other mitochondrial substrates. Sulfide induced respiration was completely abolished by inhibitors of complex III and IV, and the uncoupler 2,4-dinitrophenol blocked ATP production.
Conclusion: These results clearly demonstrate that glutamate protects cytochrome oxidase from sulfide inhibition. Thus, mitochondria are presumably more sulfide resistant in a cellular context than previously thought. Rates of sulfide oxidation can be regulated, which is an interesting new aspect regarding its role as a signal molecule. Defects in this mechanism possibly contribute to the accumulation of endogenous sulfide that is a feature of some diseases.

YSF–37
Surface mapping of cystathione beta synthase: insight into enzyme autoinhibition using mass spectrometry

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Cystathionine β-synthase (CBS) is a tetrameric enzyme containing 551 amino acids, which catalyzes condensation of serine with homocysteine. Sequence of CBS can be divided to three regions: N-terminal part (1–39), active core (40–413) and C-terminal part (414–551); Interaction of active core with C-termain domain causes enzyme autoinhibition. The 3-D structure was determined only for the truncated CBS lacking C-terminal part (amino acids 1–413, trCBS) since full-length CBS protein (wtCBS) could not be succesfully crystallized. The aim of this workis to describe molecular mechanism of the autoinhibition using the chemical modification of surface exposed amino acid residues followed by mass spectrometric detection. Initially, we tested eight labelling compounds and six of them were suitable since they have not altered the quarternary structure and activity of CBS, namely 4-hydroxyphenylglyoxal (HPG), N-ethylmaleinimide (NEM), diethylpyrocarbonate (DEP), N-hydroxysulfosuccinimideacetate (NHS), N-brom-succinimide (NBS) and N-acetylimidazole (NAI). In our ongoing study, we have analysed reactivity of four agents (NEM, DEP, NBS, NAI) with trCBS and wtCBS. Cluster of three tryptophane residues (Trp408–Trp410) was differentially reactive with NBS, modified in trCBS but not in wtCBS, indicating that the cluster is sterically hindered in wtCBS. Contradictory, cysteine (reacted with NEM), histidine (DEP) and tyrosine (NAI) modification sites were identically localizedin both forms of CBS. These data shows subtle differences in surface of trCBS and wtCBS and the modular character of the enzyme. Futhermore, this data set provides the restrains for computation modelling which would have explained the molecular mechanism of CBS activity regulation.

YSF–38
Localization and function of neuropeptide galanin in the human hair

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Galanin, a trophic factor of the central and peripheral nervous system, has been shown to have a widespread distribution in the skin, including the interfollicular and follicular epidermis. However, the exact localization and the role of galanin in the hair follicle (HF) has not been investigated. Therefore, we have determined the cellular localization of galanin in human HFs and the effects of galanin on normal growing human scalp HFs in organ culture. Immunohistochemistry was performed on cryosections of human female scalp skin. Human anagen HFs were isolated and cultured up to 9 days and treated with 100 nM galanin. Staining for Ki67, TUNEL and Masson-Fontana were used to analyze proliferation, apoptosis, staging and pigmentation of the HFs. Quantitative real-time PCR (qRT-PCR) was performed with RNA from HFs to assess the mRNA expression of galanin and the galanin receptors GALR1-3. Galanin-like immunoreactivity (galanin-LI) was detected in the outer root sheath and inner root sheath (epidermal origin) of the HFs, whereas the mesodermal connective tissue sheath and the dermal papilla were galanin-LI negative. Galanin treated organ-cultured normal human scalp HFs revealed less proliferation of hair matrix keratinocytes compared to untreated controls. Interestingly, the number of apoptotic cells was not increased in galanin treated cultures. Galanin also reduced the duration of the hair growth phase (anagen) and the melanin content after 5 and 9 days *in vitro*. The reduced hair shaft enlongation was accompanied by the development of a catagen-like morphology in hair bulbs of HFs treated with galanin, as evidenced by a round dermal papilla and decreased area containing Ki67 positive proliferating keratinocytes. The shift toward a catagen-like morphology was significant as shown by quantitative hair cycle histomorphometry. RT-PCR analysis revealed expression of GALR2 and GALR3 but not GALR1 indicating a non-GALR1 mediated function of galanin on HF growth. Thus, we present for the first time that human HFs are a source and target of galanin, and we demonstrate that galanin modulates multiple hair biology parameters, ranging from HF elongation and cycling to pigmentation.
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YSF–39
DNA-PK and PARP-1, novel interaction partners of Ets-1 transcription factor

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Ets-1 is the founding member of the Ets family of transcription factors, which are characterized by a well-conserved DNA-binding domain, called the ETS domain. It recognizes specific DNA elements, called Ets binding sites (EBS) that are present in the promoters of its target genes. This factor controls genes implied in various biological and pathological processes, such as development, haematopoiesis, angiogenesis, apoptosis and tumor invasion. In human, two isoforms of the Ets-1 protein were described: a majority isoform, the full-length Ets-1 p51, and Ets-1 p42 which results from an alternative splicing. A third isoform, Ets-1 p27, was recently discovered by our team. It is also generated by alternative splicing and acts as a dominant-negative towards Ets-1 p51-mediated transcriptional trans activation. Ets-1 regulates transcription of its target promoters via interactions with other nuclear proteins and transcription factors according to the cellular context. Moreover, it is a nuclear target of many signal transduction pathways. Identification of new proteins interacting with Ets-1 should permit to better understand molecular mechanisms involved in the regulation of its activity. For this purpose, we used an affinity purification strategy of Ets-1 partners using streptavidin pull-down. This approach requires recombinant biotinylated Ets-1 isoforms, produced using a prokaryotic expression system that we developed. Using MALDI-TOF mass spectrometry, several potential interaction partners were identified. Among those, we have determined the Poly (ADP-Ribose) Polymerase-1 (PARP-1) and the DNA-Dependent Protein Kinase (DNA-PK) complex, including its regulatory heterodimers, Ku70/Ku80 and its catalytic subunit, DNA-PKcs. The interaction between these proteins and endogenous Ets-1 were confirmed by co-immunoprecipitation in the cell. Previous studies have revealed that besides its role in DNA repair pathway, DNA-PK phosphorylates *in vitro* a number of transcription factors, such as p53, c-Fos, and c-Jun. Interestingly, *in vitro* phosphorylation assays revealed that DNA-PK was able to phosphorylate Ets-1. The role of PARP-1 on Ets-1-containing DNA-PK complex is not yet well elucidated, but we suggest that it could stimulate DNA-PK kinase activity in Ets-1 phosphorylation. Thus, DNA-PK and PARP-1 by modulating Ets-1 phosphorylation might be involved in regulation of its transcriptional activity.

YSF–40
Different methods of high-level production of therapeutic peptides in *Escherichia coli*

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Position of peptide and protein drugs grows stronger and stronger in pharmaceutical industry and has an undisputed place alongside many therapies, for certain indications they even are the only effective therapy. Biopharmaceuticals cover many therapeutic areas including mainly treatment of cancer and autoimmune diseases but also others. Originally, therapeutic peptides and proteins were extracted from natural sources but then their production has shifted to new and more advantageous biotechnological approaches such as recombinant DNA technology. These new techniques moreover allow engineering of peptides and proteins for optimal pharmacological properties. The main goal of our work is to evaluate several expression systems (mainly bacterial) suitable for production of therapeutic short peptides and to identify the best method for high-level expression at low cost. The production of biologically active peptides in bacteria (*Escherichia coli*) unfortunately has met limited success due to the low yield, presumably related to the rapid intracellular degradation of the peptides, as well as the difficulty in purification from contaminating proteins and peptides. Several methods leading to increased yield have been used. One method relies on the use of fusion partners [e.g. glutathion-S-transferase (GST), maltose binding protein (MBP) and others]. By including an appropriate protease recognition sequence, the peptide can be separated from the fusion partner by proteolytic fission (e.g. by enterokinase, factor Xa). Another method involves gene polymerization. Here, the gene of interest is expressed and purified as polymer and subsequently cleaved into monomers (e.g. by CNBr). We have developed and compared several bacterial expression systems for high-level production of human peptide hormone using the approaches mentioned above [the gene polymerization strategy, using a fusion partner (MBP) and production of the peptide alone without any fused partner or gene polymerization]. The gene polymerization strategy as well as the technique based on use of a fusion partner appeared as rapid and efficient procedures for production of varied therapeutic and also other peptides in *E. coli*, neverthless the highest yield was obtained using the method of gene polymerization.

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YSF–41
Potassium ion channels in the mitochondria from embryonic hippocampal neurons

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Recently, it has been shown that potassium transport, via ion channels, through the mitochondrial inner membrane can trigger cytoprotection. Until now four different mitochondrial potassium channels have been reported: ATP-regulated (mitoK_{ATP}), large-conductance calcium activated (mitoBK_{Ca}), voltage-dependent (mitoKv1.3) and TASK-3. It is suggested that the transport of potassium ions into mitochondria causes changes in the volume of the mitochondrial matrix, inner membrane potential, respiration, generation of reactive oxygen species and calcium influx. These changes most likely occur in order to protect the cell from death. In our study a single channel activity was measured with the use of patch-clamp of the mitoplasts isolated from embryonic hippocampal neurons. Our data provides evidence for the presence of mitoBK_{Ca} channels in the inner mitochondrial membrane of rat hippocampus. The channel conductance calculated based on current-voltage relations was equal 289 pS. The activity of the channel deceased at low calcium concentration. The effect was reversed after application of NS1619, an activator of the BK types channels. Additionally, channel activity was blocked by paxilline (inhibitor of the BK types channels). We also identified

a novel channel which has current-voltage characteristics similar to the inwardly rectifying potassium channels. Patch-clamp studies showed that this channel is not sensitive to the known activators and inhibitors of mitoBK$_{Ca}$, mitoK$_{ATP}$ and mitoKv1.3 channels. In summary, the findings presented in this study provide new functional data suggesting the presence of the two channels in the rat hippocampus mitochondria: BK$_{Ca}$- type and inwardly rectifying potassium channels.

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YSF–42
The influence of chain shuffling on the properties of anti-aflatoxin antibodies

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Today antibody-based assays are among the most effective techniques available for the measurement and detection of toxins, poisons, drugs. However, the performance of these methods strongly depends on the specificity and the sensitivity of the antibodies used. Aflatoxins, metabolites produced by fungi *Aspergillus flavus* and *A. parasiticus* often contaminate food and feed crops and have been classified as group I carcinogens by the International Agency for Research on Cancer. The molecules of aflatoxins are of small size, and as a consequence, most of monoclonal antibodies to these antigens have moderate affinity. The present work is aimed at the development of recombinant antibodies (rAb) against aflatoxins with changed specificity and sensitivity. After the detailed analysis by immunological methods 10 monoclonal antibodies (mAb) against aflatoxins B1, B2 and G1 were selected for the protein engineering studies. Their affinity and cross-reactivity to the different types of aflatoxins were characterized by indirect and competitive ELISA. Total RNA, isolated from hybridoma cells producing these mAbs, was used for subsequent cDNA synthesis and amplification of Ab gene fragments. Sequence analysis of the immunoglobulin heavy and light chain variable regions showed that the mAbs have different heavy chains, but only two types of light chains. It can be inferred from these findings that the difference in mAbs affinity and cross-reactivity is provided by their heavy chain structure. Based on these observations, antibody light and heavy chain shuffling was proposed as a strategy leading to the development of rAbs with altered binding parameters. Native and shuffled antibodies were expressed in the form of Fab fragments in *E. coli* cells. Fab fragments obtained by virtue of enzymatic hydrolysis of full-size anti-aflatoxin antibodies have been used as controls in the study of affinity and cross-reactivity of expressed rAbs. The functional characteristics of obtained rAbs were tested in direct, indirect, and competitive ELISA relative to different aflatoxins and aflatoxin conjugates.

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YSF–43
Immunization with a synthetic fragment of the alpha7 nicotinic acetylcholine receptor causes therapeutic effect in mice with experimentally induced Alzheimer's disease

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Alzheimer's disease (AD) is an incurable neurodegenerative disorder that produces cognitive impairments that increase in severity as the disease progresses. Current AD therapeutics provides mainly symptomatic short-term benefit, rather than targeting disease mechanisms. One of the hypotheses of AD neuropathology involves high affinity binding of beta-amyloid with the alpha7 nicotinic acetylcholine receptor (AChR) leading to neuronal lysis. We investigate a new way of AD treatment. We proposed that antibodies against alpha7 AChR would prevent the receptor from binding with beta-amyloid and the development of neurodegenerative process. In our experiments we used bulbectomized (BE) mice which had olfactory bulbs removed as a model of sporadic AD. Protective effect of immunization with synthetic fragment 173–193 of alpha7 AchR was studied on BE and non-bulbectomized, so called sham-operated (SO) mice. Synthetic fragment 171–199 from meningococcal protein OMP1 and the keyhole limpet hemocyanin (KLH) conjugate of synthetic fragment 197–213 of foot-and-mouth disease virus protein VP1 were used as negative control compounds. After double immunization 82% of BE mice immunized with alpha7 173–193 peptide demonstrated good spatial memory in Morris water maze while the spatial memory of BE mice immunized with control compounds proved to be impaired. All SO mice immunized with alpha7 173–193 peptide as well as mice immunized with OMP1 fragment and KLH conjugate of VP1 fragment revealed no spatial memory impairment. ELISA showed high levels of antibodies in the blood sera of BE and SO mice immunized with 173–193 peptide and with control compounds. Antibodies to all the fragments were detected in the cerebrospinal fluid of BE animals but they could not penetrate the blood-brain barrier in SO mice. BE mice immunized with alpha7 173–193 peptide had the decreased level of brain beta-amyloid to 11 ng/g in comparison to 38 ng/g in BE mice immunized with control OMP1 fragment and KLH conjugate of VP1 fragment. To prove that mainly antibodies to alpha7 AChR caused a therapeutic effect on the memory of BE mice we used passive immunization of BE mice with blood sera containing anti-173–193 antibodies. We demonstrated antibodies to alpha7 AchR led to memory improvement. Thus, we showed that immunization with synthetic fragment 173–193 improves cognitive decline of BE mice. Antibodies to alpha7 AChR proved to cause the therapeutic effect on animals with experimentally induced AD.

YSF–44
Molecular mechanism of DNA topoisomerase II-induced chromosomal rearrangements

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Secondary leukemias constitute a serious complication of cancer chemotherapy with topoisomerase II – specific drugs. It is believed that these leukemias develop because of chromosome rearrangements originating as a result of illegitimate recombination. Topoisomerase II (topoII) may be involved in induction of illegitimate recombination either directly via subunit exchange or indirectly via introducing double-strand breaks (DSB) into DNA and subsequent activation of the non-homologous DNA end joining (NHEJ) repair system. Repair of DSB by NHEJ frequently results in illegitimate recombination. TopoII of the nuclear matrix is believed to be the main target of antitumor drugs and thus recombination junctions frequently resides within nuclear matrix association regions (MAR). We demonstrated that stalled complexes of topoII accumulated under conditions of inhibition by etoposide (VP16) are recognized as DSB by cellular DNA repair systems and are marked by phosphorylation of histone H2AX. Furthermore, the γH2AX foci remained visible at nuclear matrices and colocalized with the major components of non-homologous end joining system of DSB repair. Using chromatin immunoprecipitation (ChIP) assay we have shown that inhibition of DNA topoisomerase II in cultured cells stimulates association of components of NHEJ system with a known breakpoint cluster region (recombination hot spot) of the human AML1 gene. Preferential assembly of repairing complexes on a known breakpoint cluster region (bcr) of AML1 gene is of special importance. It may reflect the fact that being located on the nuclear matrix this bcr is preferentially cleaved by nuclear matrix DNA topoII. It is important that bcr3 of AML1 gene contains MAR and the so-called *in vivo* topoII cleavage site as was reported previously and reconfirmed in our study by PCR-stop experiments. Many other bcr (recombination hot spots) mapped in chemotherapy-related secondary leukemias also contain MARs and sites of *in vivo* DNA cleavage by topoII. Thus, error-prone repair of topoII-induced DSBs by NHEJ is a plausible cause of the recombinogenic effect of topoII-specific antitumor agents. The results obtained in this study corroborate the hypothesis that illegitimate recombination between sites of DNA loop anchorage to the nuclear matrix may contribute to the specificity of chromosomal rearrangements.

YSF–45
Influence of proteins from Agkistrodon blomhoffii ussuriensis snake venom on human hemostasis system

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Background: The venom of different snakes belonging to the Crotalidae and Viperidae families are complex mixtures of pharmacologically active proteins and polypeptides. Many of these biopolymers act upon the blood coagulation system and can be used in the diagnostic and treatment of haemostatic disorders.
Objectives: The main purpose of this work was to develop industrial scale method for purification of different proteins from Agkistrodon blomhoffii ussuriensis snake venom and investigate their influence on human hemostasis system.
Material and Methods: We developed different model systems to test the influence of proteins from the Agkistrodon blomhoffii ussuriensis snake venom on blood coagulation cascade and especially on platelet activation and aggregation. The systems were both done *in vitro* using coagulometry, aggregometry and flow-cytometry analysis. Molecular masses and the purity of the proteins were detected by 2D-SDS-PAGE and HPLC.
Results: Low molecular weight disintegrin, phospholipase A_2, protein C activator, fibrino(geno)lityc and α-specific thrombin-like enzymes were purified from Agkistrodon blomhoffii ussuriensis venom in industrial scale using affinity, ion-exchange and hydrophobic chromatography. Low molecular weight disintegrin and PL_2 isolated from this venom activate platelets and strongly inhibit their ADP- and adrenalin-induced aggregation in distinct ways. Fibrino(geno)lityc enzyme which predominantly cleave α-chain of fibrinogen does not activate platelets and has no effect on the aggregation stimulated by collagen, but on the other hand it does inhibit ADP and adrenalin-induced platelet aggregation. Thrombin-like enzymes, activate washed human platelets but have no effect on the aggregation in the absence of fibrinogen.
Conclusions: The obtained results make it possible to draw the conclusion that purified proteins can be as an instrument under investigation of protein-protein interactions in haemostasis system and for the development of new cardiovascular therapeutic agents.

YSF–46
Computational studies of substrate binding and alternating access in the glycine betaine:sodium symporter BetP

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The glycine betaine symporter BetP is an important protein for the regulation of osmotic pressure in *Corynebacterium glutamicum*. The recently solved X-ray structure of BetP reveals that it is a homotrimer and that each protomer possesses its own substrate binding pocket. The BetP protomers adopt a similar fold as that found for several other secondary transporters, and these appear to correspond to different states in the transport cycle. However, no high-resolution structures have been reported for the same transporter in two alternate states. All three protomers in the BetP X-ray structure show only narrow substrate pathways from the cytoplasmic side, and thus are likely to represent an inward-facing occluded state. To understand the alternating-access mechanism of BetP, we have constructed its 3D models using secondary transporters of known structure as templates and validated modeling results through fitting of the models to cryo-EM maps of BetP. In these maps the protomers within the same trimer appear to represent three distinct states, probably as a result of the truncation of the C-terminal domains involved in the regulation mechanism. In addition, we have performed MD simulations of BetP to address several other questions, including: the location of Na^+ binding site, the effect of the charge on the lipid headgroups, and the importance of the trimeric state of the protein. Finally, we combined the results of structural and simulation studies with those from so-called correlated mutation analysis of sequences of related transporters to identify structural/functional roles for several important residues. We found that the presence of residues crucial for the formation of the trimeric state (identified by virtual alanine scanning) correlates well with the presence of residues stabilizing the position of the C-terminal domain. This result suggests that formation of the trimer is indeed critical for a regulation mechanism in which the C-terminal domains interact with adjacent protomers. The results of our

computational studies lead to a better understanding of key events in transport and regulation, and are currently being validated experimentally.

YSF–47
Analysis of species diversity of the tomato pathogens in greenhouse farms in Belarus

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Indoor cultivated tomato is one of the most economically important crops for Republic of Belarus. This crop is highly susceptible to diseases of various nature, especially bacterial infections. Currently, there is a significant lack of research concerning tomato bacterial infections. This limits assessment of current phytopathologic states within the communities of these crops. The main objective of this investigation was to estimate the phytosanitary conditions of tomato indoor communities in a range of greenhouse farms in 2008. This included the assessment of the presence of bacterial infections and species diversity of the pathogens. Vegetating plants were observed in nine greenhouse farms throughout the year. Upon the detection of bacterial infection samples were taken for laboratory analysis. Microorganisms were purified from the infected samples using standard microbiology techniques. Purified cultures were tested for phytopathogenic properties by injection into the leaf tissue of broad beans (Vicia faba). The species were identified by biochemical tests and PCR-analysis with specific primers recommended in literature for the detection of phythopathogenic *Clavibacter michiganensis* subsp. michiganensis, *Pseudomonas corrugata* and *Pseudomonas mediterranea*. Nucleotide sequence of the 16S RNA from the purified clones was also determined and compared to known sequences in the BLAST database. Overall, 24 infected samples were obtained from nine farms. Among the microorganisms purified 23 phythopatnogenic strains were selected. According to a range of physiological and biochemical tests, 10 of them belonged to a single species of gram positive microorganism. Using PCR these strains were identified as *C. michiganensis* subsp. michiganensis, a pathogen of tomato bacterial cancer. Outbreaks of this disease were registered at seven out of nine farms observed. The other species are currently being identified by sequencing of 16S RNA gene. In conclusion, the up-to-date analysis of the pathogen diversity suggests that bacterial cancer was a prevalent tomato disease within a range of the observed greenhouse farms in Belarus in 2008.

YSF–48
New action mechanisms for ADNP/NAP as immunomodulatory players.

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ADNP (activity-dependent neuroprotective protein) has a wide distribution in Central Nervous System (CNS) with an important function on genes associated with neural tube development during embryogenesis. ADNP harbors neuroprotective activities that relay on the ADNP-derived octapeptide sequence called NAP. We identified for the first time NAP as an immunoregulatory molecule. We investigated NAP's potential in experimental animal models of neuroinflammation to explore NAP effects in CNS. The secretion of proinflammatory cytokines, IL-6 and TNF-α, were measured by ELISA and IL-6, TNF-α, CD3 and CD11b expression by RT-PCR. Suppressive activities of NAP on innate and adaptative immunity were found. The expression and secretion levels of these cytokines and inflammatory markers were lower in NAP treated animals. NAP immunomodulatory activities were also analyzed in primary mixed cultures where NAP activity was similar *in vivo* and in primary mixed cultures. To test the physiological role of ADNP in immune regulation *in vivo*, we used heterozygous ADNP KO ($Adnp^{+/-}$) mice in a model of CNS inflammation induced by intra-ventricular injection of lipopolysaccharide (LPS). Following a challenge with LPS, $Adnp^{+/-}$ mice showed an increased expression and secretion of IL-6 and increased numbers of activated microglia and infiltrating leukocytes compared to wild-type (WT) mice. An increase in class II transactivator (CIITA) mRNA levels suggests that the functions of major histocompatibility complex (MHC) II molecules in antigen processing cells (APCs) could be affected in the $Adnp^{+/-}$ mice. This was accompanied by impaired IL-10 secretion in LPS-treated $Adnp^{+/-}$ mice compared to WT. These remarkable characteristics of NAP are important in future treatments of CNS pathologies with inflammation components, for example, Alzheimer's disease, Parkinson disease or multiple sclerosis.

YSF–49
Replication timing of genomic domains correlates with their DNase sensitivity

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Background: Replication of each genomic locus proceeds through a temporally ordered process. Almost all housekeeping genes replicate within the first half of S phase in many cells. Tissue specific genes have a developmentally regulated pattern of replication whereby they undergo DNA synthesis early in expressing cells, but late in non-expressing cell types. Using differently organized chicken alpha- and beta-globin gene domains as a model system we tried to ascertain its replication timing pattern in cells of different origin.
Methods: We have used fluorescence in situ hybridization as a conventional tool for studying the replication timing of specific genomic loci. To characterize the chromatin configuration of these loci we have quantitatively evaluated DNase I sensitivity as the extent of DNA digestion quantified by real-time polymerase chain reaction.
Results: We have shown that chicken beta-globin domain is early replicating in chicken erythroleukemia cells, but late replicating in cultured chicken lymphoblasts and fibroblasts, while alpha-globin genes, which are expressed in a strictly tissue- and developmental-stage specific manner, replicate early in both erythroid and non-erythroid cells. The reason for that fact seems to be the presence of early replicating domain of housekeeping gene overlapping domain containing alpha-globin genes. That is confirmed by the selective DNase-sensitivity of beta-globin gene domain dependent on the expression profile and general DNase-sensitivity of alpha-globin gene domain independent on the gene expression.
Conclusions: We assume that the pattern and timing of replication of the precise gene locus is more dependent on the underlying chromatin structure rather than on its transcriptional activity. That could be explained by increased accessibility of potential replication origins to the replication machinery by the constitutively open chromatin environment.

YSF–50
Artificial enzymes as potent tools in molecular biology

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Small synthetic compounds capable of function as natural enzymes are called artificial enzymes. One of the examples is organic compounds, cleaving RNA with high efficiency – artificial ribonucleases. Usually they contain groups found in active sites of natural enzymes. Such a small molecule, capable of nonspecific interactions with RNA and inducing its cleavage, could be used for examination of RNA three-dimensional structure and its conjugation to antisense oligonucleotides opens up possibilities of development of site-directed conjugates. New type of artificial ribonucleases: conjugates of cationic and hydrophobic fragments were developed, synthesized and examined. It lacks traditional functionalities known to catalyze transesterification reaction and consists of two positively charged bisquaternized 1,4-diazabiciclo[2.2.2]octane residues substituted with lipophilic fragments and connected by rigid aromatic linker. The ribonuclease activity of combinatorial libraries of these compounds was examined and the most active individual compound, named Dp12 (contain two dodecyl groups) was identified. The mechanism of RNA cleavage includes RNA conformation rearrangements via hydrophobic interactions after Dp12 binding to RNA. Dp12 form complex with every phosphodiester bond and catalyze transesterification due to the bending conformation of ribosophosphate backbone, that makes easier self-hydrolysis of RNA in water solution. The potential of Dp12 as antivirals was studied. We demonstrated that Dp12 is able to inactivate influenza virus. Treatment of influenza virus prior to infection entirely destroys viral genomic RNA and prevents development of virus infection in MDCK cells, in embryonated chicken eggs and in mice. Thus, Dp12 represent a new class of compounds capable of inactivation of RNA containing viruses.

YSF–51
Bcr-Abl influences the p53 k317 acetylation and localization protecting from bax activation and DNA damage-induced apoptosis

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Post-translational regulation of p53 is an important way to regulate its role, and p53 acetylation is responsible for molecular switch between survival and cell death. The lack of K317 p53 acetylation leads to p53 translocation to the cytoplasm followed by Bax activation and induction of apoptosis. Previously, using the mouse model of CML, that is 32D progenitor cell lines without and with different levels of Bcr-Abl, we showed that high expression of Bcr-Abl, protected cells from Bax translocation to the mitochondria and cell death in response to DNA damage. Now, we found that Bcr-Abl expression correlated with increased p53 K317 acetylation and nuclear localization. After etoposide treatment all cell lines showed the same level of DNA damage and p53 Ser15-P, but Bcr-Abl-expressing cells were less sensitive to apoptosis. p53 K317 acetylation was higher in cells with Bcr-Abl. Moreover, in Bcr-Abl-expressing cells, p53 stayed in the nucleus. It correlated with protection from activation of Bax. Bcr-Abl inhibition decreased the p53 K317 acetylation and led to the p53 translocation to the cytosol, Bax activation and finally cell death. These results imply that Bcr-Abl expression affects the regulation of p53 acetylation in response to DNA damage, p53 translocation to the cytoplasm and Bax activation thus protecting from apoptosis. We postulate that the Bcr-Abl-mediated influence on the p53 acetylation could be a significant factor involved in the development of apoptosis resistance and disease progression.

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YSF–52
Correlation between the increase of lectin activity and complication of benign prostate hyperplasia

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Galactose-specific lectins intensively involve in the transformation process and participate in the various biological processes. We have studied the spectrum of lectin activity in subcellular fractions of human prostate tissues at different pathologies. Post-operational prostate tissues with following pathological forms: BPH-benign prostate hyperplasia, PIN-prostate intraepithelial neoplasia, AAH- atypical adenomatous hyperplasia was used as experimental material. For isolation of membrane lectins, 0.5%-solution of the detergent Triton X-100 was used. Isolation/purification of galactose-specific lectins was made on the trisacryl-d-galactose columns. Hemagglutination activity of the lectins was assessed with 2% suspension of the rabbit's trypsinized erythrocytes. Membrane proteins with lectin activity have been revealed in subcellular fractions (mitochondria, microsome, plasma membrane, nucleus) of human BPH, PIN and AAH tissues; their specific activities were differ and AAH. Change of → PIN → increased sharply with following directions: BPH carbohydrate-specific spectrum of lectins in all subcellular fractions was designated in relative of different pathological forms. Especially, it has been exposed the correlation between increase of galactose-specificity and complication of disease. Therefore, we have isolated the galactose-specific lectins (Gal-lectins) from mitochondria and microsome of all pathological tissues. Despite of identical molecular weights (60 kDa), it has been revealed the significant increase of specific activity of both mitochondrial and microsomal Gal-lectins with complication of prostate hyperplasia. Therefore, during complication of disease the biological activity of lectins is changed. It is possible that alterations of these features are accompanying of the process of transformation, but in this case these alterations aggravate those biochemical processes, which provide the neoplasic growth of cells.

YSF–53
Differential modulation of the neuroinflammatory response by wild-type and mutant α-synuclein

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Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized pathologically by the presence, in the brain,

of intracellular protein inclusions highly enriched in aggregated α-Synuclein (α-Syn), known as Lewy Bodies (LB). The onset of disease has been shown to be accompanied by a local immune reaction in regions of the brain affected by the LB, and its progression is characterized by sustained activation of microglia, which is linked to significant dopaminergic neuron loss. Traditionally, α-Syn has been viewed as an exclusively intracellular, cytoplasmic protein. Lately, accumulating evidence showing the release and exocytosis of α-Syn to the medium has pointed at the importance of studying the effects of extracellular α-Syn on surrounding cells in the brain. However, detailed information about its effect on cellular and molecular components of the immune system, and its link to PD pathology, is still very scarce. In this work, we aimed at assaying the immunomodulatory capacity of extracellular α-Syn, both wild-type and familiar PD-related α-Syn mutants A53T, A30P and E46K. For this, we used mouse primary mixed cultures, as well as isolated macro and microglia. After stimulating cells by adding various concentrations of α-Syn and mutants, we systematically measured the release by these brain cells, of key interleukins (IL-6, IL-10, IL-1β, IL-17) and chemokines (MCP-1, RANTES, IP-10), by ELISA. Interestingly, we have found remarkable differences in the cytokine expression profiles between wild-type and the different mutants. These findings are important since, as opposed to previous works, primary cultures rather than cell lines, and no co-stimulation, were used in the assays. In particular, we observed that, while some α-Syn variants exhibit a primarily immunostimulatory effect, other mutants induce an essentially immunosuppressive response. These results give us possible clues about the origins, progression and potential therapeutic approaches towards this widespread and highly devastating disease.

YSF–54
Oxidized low density lipoprotein treatment modifies the expression of some JAK-STAT signaling pathway genes

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Background: Oxidative modification of low density lipoprotein (oxLDL) is one of the earliest events in atherosclerosis. This pathology is known as a chronic and inflammatory process that is characterized by the accumulation of lipids, inflammatory cells, and fibrous tissue. All together lead to a thrombotic process in arteries that start at the beginning of the life. Human umbilical arterial endothelial cells (HUAEC) are a good model to study these first steps of this disease. Due to the impact of this pathology in the society there is a need to improve the knowledge about it.
Objectives: Our aim was to analyze the effect of oxLDL on gene expression profile in HUAEC, looking for new genes involved in the atherosclerotic process.
Methods: HUAEC were exposed to oxLDL (100 μg/ml) for 24 hours. Data obtained from Genome U133 Plus 2.0 microarray were analyzed with Gepas, Bioconductor, and FatiScan software. Biological interpretations were obtained with Ingenuity Pathway Analysis and Pathway Studio. Microarrays validation experiments were performed with Real time PCR using TaqMan assays. Additional Western blot experiments were done to ensure the results.
Results: Treatment with oxLDL affected the expression of 201 probes: 142 were up-regulated and 59 down-regulated. The most altered categories were lipid metabolism, immune response and atherosclerosis induction functions. Many of the most up-regulated genes were part of the JAK-STAT signalling pathway and interferon induced proteins.
Conclusions: In this study we provide solid evidences about the gene expression regulation modulated by oxLDL in endothelial cells. This fact could be an important start point for future therapies against atherosclerosis.
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YSF–55
Construction and characterization of recombinant antibodies against Tick-borne encephalitis virus

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Earlier a panel of mice hybridoma cell lines, producing Mabs against glycoprotein E of Tick-borne encephalitis virus (TBEV) was generated, from which several Mabs demonstrated neutralizing and protective activities. 13D6 Mab with neutralizing and protective properties was used as the parental antibody for scFv and chimeric antibodies construction. In order to develop scFv-antibody the total RNA was extracted from hybridoma cells and used as template for RT-PCR to obtain the VH and VL cDNAs. These variable regions were then assembled in scFv structure by PCR using a (Gly4Ser)3 linker and ligated into the pGEM1 vector. The resulting plasmid pSC13D6 provided an expression of scFv-antibody against TBEV in *Escherichia coli* cells. As expected, ELISA analysis showed that cell lysates containing scFv13D6 exhibit specific binding to TBEV (strain 205). After purification of scFv-antibody by gel filtration the affinity constant of this antibody was measured. Affinity of purified scFv13D6 was (3.0 ? 0.2) ? 107 M-1. The neutralizing activity of scFv-antibody was tested using focus reduction-neutralization test and FRNT50 was estimated. FRNT50 of scFv13D6 against TBEV strain 205 was determined as 16.7 ěg/ml. Further, on the basis of the V-genes of scFv13D6 we have constructed recombinant plasmids pD6H and pD6L, encoding heavy and light chains of fully mouse-human chimeric antibody against TBEV. The joint transfer of these plasmids into HEK 293T cells resulted in the production of chimeric antibody. After purification of chimeric antibody by affinity chromatography the affinity constant of this antibody was measured. Affinity of purified chimeric antibody 13D6 was (7.0 ? 0.3) ? 107 M-1. At present we investigate the neutralizing properties of chimeric antibody in focus reduction neutralization test.

YSF–56
Trapping molecular metamorphosis

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Background: The birch pollen allergen Bet v 4 represents a large family of structurally related allergens. While detailed

structural information is available on this protein family, the distinguishing structural features that render this protein allergenic and enable it to crosslink IgE antibodies remain poorly understood. This situation is reflected by the lack of reliable structural motifs that could serve as hallmarks of allergenicity, such as a catalytic triad and oxyanion hole identify a protease.

Objectives: Our working hypothesis is that the allergen Bet v 4 – as well as structurally related allergens – distinguishes from non-allergenic proteins by their ability to adopt different conformations, including different oligomerisation states. We aim at (i) identifying physico-chemical parameters that govern the conformational transitions and allow to stabilise distinct molecular conformations. Further, (ii) we aim to characterize the stabilized conformational states on atomic level, thus revealing the exact mechanisms of the molecular metamorphosis in Bet v 4. This insight should enable us to actively control/inhibit oligomerisation transitions. Finally, (iii) we aim to profile the allergenic behaviour of the distinct molecular substates of Bet v 4.

Methods: In a systematic approach we screened the effect of physico-chemical parameters like salt, detergents or temperature on the oligomerisation behaviour of Bet v 4. With SDS-PAGE and gel filtration chromatography we utilise two orthogonal read out systems to characterise the oligomerisation state of the protein. Additionally, we employed ESI-MS to confirm the chemical identity of the distinct conformers. Stabilised conformers are further investigated by crystallisation and by functional studies, including IgE binding assays.

Results: By controlled temperature changes we could reversibly induce distinct conformational transitions in Bet v 4, a behaviour which we refer to as molecular metamorphosis. We further succeeded to covalently stabilise selected conformers; these locked conformers allow now for further detailed structural and functional studies.

Conclusions: These investigations may result in nothing less but the identification of a unifying architectural principle of allergens.

YSF–57
FMO3 polymorphisms in 13 ethnic populations: frequency and linkage analysis

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Background: Catalytic efficiency of the drug metabolizing enzyme FMO3 can be altered due to genetic variations. A decreased functional activity is associated with several single nucleotide polymorphisms (SNPs) (E158K, V257M, E308G and D132H) whereas only one (L360P) causes activity increase. Knowledge of population frequencies is pivotal to estimate clinical relevance of the SNPs.

Objectives: To evaluate the prevalence of three wide-spread (E158K, V257M, E308G) and two African specific FMO3 SNPs (D132H, L360P) in 13 different ethnic groups.

Methods: Allelic frequencies were determined by TaqMan allelic discrimination assay in 2152 healthy volunteers from Europe (Swedes, Italians, Turks), East Asia (Japanese) and sub-Saharan Africa (nine ethnic groups covering Eastern, Southern and Western regions) followed by linkage analysis. Individuals identified by TaqMan as carriers of L360P were further sequenced for verification.

Results: Significant regional differences ($p < 0.001$) in allelic frequencies were found for E158K, V257M and E308G within Europe and for D132H within sub-Saharan Africa. None of the 863 Africans carried P360 variant which questions its qualification as polymorphism. The frequencies of M257 and G308 among sub-Saharan Africans were significantly lower than previously reported for African Americans. K158 and G308 was cis-linked (haplotype K158/G308) in all populations with high proportions (12.0–38.3%) of carriers in non-African groups but rare among Africans (0–1.6%).

Application to practice: As haplotype K158/G308 significantly affects FMO3 enzyme activity towards several commonly used drugs, knowledge of the population frequencies and awareness of large interethnic differences can help clinicians to design individualized treatment dosage of these drugs.

YSF–58
Inflammatory potentiation of TRPV1 channel through regulated exocytosis in nociceptive neurons

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Background: Potentiation of the pain-integrator termoreceptor TRPV1 underlies thermal hyperalgesia mediated by different pro-inflammatory factors including NGF, BK, IGF-I, ATP, IL-1b, and ART. Diverse mechanisms for inflammatory sensitization of TRPV1 have been proposed, namely a decrease in its activation threshold and an increment in its surface expression in nociceptors. However, the molecular mechanism underlying the inflammatory-induced membrane translocation of TRPV1 in DRG neurons remains elusive.

Objectives: We investigated the contribution of regulated exocytosis to the inflammatory sensitization of TRPV1 in neonatal DRG neurons. We evaluated the effect of compound DD04107, a small peptide patterned after the N-terminus of the SNARE protein SNAP25, which selectively inhibits regulated exocytosis, on the capsaicin-induced Ca^{2+} influx and the receptor surface expression by means of Ca^{2+} imaging and immunocytochemistry assays.

Results: We demonstrate that the enhancement of TRPV1-mediated Ca^{2+} fluxes provoked by NGF, ATP and IGF-I was strongly blocked by peptide DD04107. The increase in the surface expression of TRPV1 potentiation induced by these compounds was fully prevented by treatment with compound DD04107. In contrast, TRPV1 sensitization caused by BK, IL-1b and artemin was insensitive to the SNARE blocker.

Conclusions: These results indicate that some, but not all, pro-inflammatory agents induce the recruitment of TRPV1 channels to the neuronal surface. Therefore, inflammatory potentiation of TRPV1 in DRG neurons is contributed by both the fusion of a vesicular population of receptors and the modification of the channel. These findings support the tenet that SNARE-dependent exocytosis of TRPV1 is a valid therapeutic target to treat inflammatory pain.

YSF–59
Regulation of hypericin photodynamic action mode by antioxidants

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The key component of H. perforatum extracts – hypericin (HY) is considered as a potent blood sterilizer and photosensitizer for

application in photodynamic therapy. In the present work the influence of antioxidants on HY photodynamic action was studied that indirectly will provide better understanding of mechanisms of HY photodynamic action. As a model system HY induced erythrocyte photohemolysis was used (1). There were used ascorbic acid (0.15–7.5 μM), tryptophan (1–30 mM, Aldrich, Germany) and quercetin (10^{-7}–10^{-4} M, Roth, Austria) as antioxidants. Erythrocytes were exposed to visible light of filament lamp (30 mW/cm^2) in the presence of HY (0.28 μM Roth, Austria) with or without antioxidants. Photohemolysis degree was assessed by absorbance changes at 680 nm by Specord M400 (Carlzeiss, Germany). It was revealed that depending on concentration quercetin, ascorbic acid and tryptophan suppress or stimulate photohemolysis induced by HY with the same tendency. Thus photodynamic action of HY, beside singlet oxygen, is mediated via other reactive oxygen species. Strengthening of photohemolysis by antioxidants in higher concentrations indicates the switching of alternative mechanisms of HY photodynamic action and its complicated manner. It could be concluded that the photosensitization reaction realized by HY depends on antioxidant concentration and could be shifted. This may be useful for further clinical application of HY together with antioxidants.

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YSF–60

Effect of oxidative phosphorylation inhibitors on the electromechanical activity in rat and human myocardium

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Background: The main function of heart – contraction – is strongly dependent on energy (ATP) generated by mitochondrial oxidative phosphorylation which comprises the respiratory chain (MRC) and F_1F_0-ATPase. During heart failure F_1F_0-ATPase starts to hydrolize ATP and becomes the main consumer of energy. The inhibition of this enzyme could be one of the ways to stop ATP wasting and to preserve energy in failing myocardium.

Objectives: We investigated the effect of inhibitors of MRC complexes III (antimycin A) and IV (anoxia) alone and under the inhibition of F_1F_0-ATPase by oligomycin on the electromechanical activity in rat and human myocardium.

Methods: Rat heart papillary muscles and human ventricular strips from patients undergoing corrective open-heart surgery were used. Experiments were performed using standard method of registration of myocardium electromechanical activity and the whole cell patch-clamp technique for registration of L-type Ca^{2+} current (I_{CaL}) in single cardiomyocytes.

Results: Antimycin A (10^{-8}–3×10^{-4} M) and anoxia (3 mM $Na_2S_2O_4$) caused reduction of contraction force (P) of rat and human myocardium and I_{CaL} of cardiomyocytes. Oligomycin (2×10^{-5} M) reduced the suppressive action of antimycin A on P 3-fold and 1.5 fold in rat and human myocardium, respectively, and removed its inhibitory effect on I_{CaL} in human cardiomyocytes. The considerable protective effect of oligomycin during anoxia was established also.

Conclusions: Suppression of F_1F_0-ATPase activity reduced the inhibitory effects of respiratory chain complexes III and IV inhibitors on electromechanical activity in rat and human myocardium.

YSF–61

Protective effects of ischemic preconditioning and pre-treatment with TNFα in a rat model of ischemia-reperfusion injury

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Ischemia-reperfusion injury (IRI) is a phenomenon whereby reestablishment of blood flow causes more severe damages than ischemia alone. IRI is found to be a reason of liver dysfunction and failure after hypovolemic shock, its resection by clamping hepatoduodenal ligament or transplantation. Thus, protecting liver against IRI becomes an important clinical problem. The aim of the study was the comparison and effectiveness assessment of two IRI preventing methods: ischemic preconditioning (IP) and pre-treatment with a low dose of TNFα. The study was performed on male Wistar rats divided into two controls (intact and laparotomy) and three experimental (ischemia, IP and TNFα pretreatment with 3 ng/kg body weight dose) groups. The blood and liver specimens were taken 1, 6 or 72 hours after ischemia. Succinate dehydrogenase, lactate dehydrogenase and glucose-6-phosphatase activity estimation, and p.a.S reaction and H&E staining were performed. The enzymes activity was evaluated densitometricaly by Image-Pro Plus software. Alanine transaminase, alkaline phosphatase, malondialdehyde concentration, FRAP-test and paraoxonase activity in plasma and myeloperoxidase, TNFα and conjugated dienes concentration in liver homogenates were also measured. Pre-treatment with TNFα was more efficient than IP in stabilizing and maintaining most of the marker enzymes activity at control values level during reperfusion. Observed morphological changes regressed more quickly after TNFα treatment. The performed evaluation suggests that the administration of a low-dose of TNFα could be more effective in the protection against IRI than ischemic preconditioning.

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YSF–62

Selection of 'better performing' laccases through directed evolution

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Fungal laccases are remarkable green catalysts that have a broad substrate specificity and many potential applications in bioremediation, lignocellulose processing, organic synthesis, and more. The white-rot fungus *Pleurotus ostreatus* is able to express multiple laccase genes encoding isoenzymes with different and particularly interesting physico-chemical characteristics: POXC, POXA1w, POXA1b, POXA3a and POXA3b (1). Several *P. ostreatus* laccases have been successfully expressed in yeasts (2) and the availability of established heterologous recombinant expression systems has allowed the construction of mutated, 'better performing' enzymes through molecular evolution techniques (3). Directed evolution has emerged as method of choice for engineering functions and properties of the analyzed enzymes.

This technique relies on Darwinian principles of mutation and selection by introducing randomly distributed mutations on the gene encoding the enzyme of interest. The resulting 'library' of mutants is screened for a desired characteristic to attempt to identify a mutant enzyme that exhibits the characteristic of interest. We successfully used two cDNAs encoding *P. ostreatus* laccases, POXC and POXA1b, as 'parent molecules' to guide the evolution of laccases. Genetic variants were created by random mutagenesis through error prone PCR (EP-PCR) and DNA shuffling. After two round of mutations, four POXA1b mutants (1M9B, 1L2B, 1M10B and 3M7C) were selected for their improved activity against ABTS. Molecular dynamic simulations have been performed on the models of POXA1b, 1M9B, and 3M7C, and their analyses suggest that the substitution P494T is responsible both for the increased stability and for the higher activity of this mutant (4). New criteria of selection were applied in a further screening of the 2400 mutants library, using different substrates (e.g. 2,6 dimethoxyphenol, DMP), assaying the enzyme stability and activity at different operating conditions (pH, temperature). Three new mutants were selected for their improved performances and characterised from a structural and functional point of view.They showed stability at pH 5 and at 60°C higher than that of the wild-type enzyme. A new mutant was rationally designed and constructed. This new mutant, R4, contains the mutations of the two parental enzymes (1M10B and 3M7C): L112F, P494T, K37Q and K51N. R4 showed stability at pH10 2 fold higher than that of the wild-type enzyme. This new clone was used as template for producing a new collection of 1100 mutants. Two new mutants (1H6C and 1M10G) were selected for their improved activity against ABTS. Thermodynamic and catalytic characterization of this mutant is in progress. This new library will be screened with different criteria of selection.

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YSF–63
The nitrite reductase activity of mammalian isoforms of nitric oxide synthases under hypoxia

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Nitrite anions (NO^{2-}) appear as metastable intermediates in the oxidation cascade leading from nitric oxide (NO) radicals to the stable metabolite nitrate that induce vasodilatation under hypoxia. We tested the *in vitro* ability of the three nitric oxide synthase (NOS) isoforms to release NO from nitrite under anoxia using electrochemical detection combined with absorption spectroscopy. The release of free NO from anoxic nitrite solutions at 15 μM was specific to the endothelial isoform eNOS and did not occur with neuronal nNOS and inducible iNOS. Our data suggest that the rate of reduction of the heme, possibly associated with structural dynamic changes in the distal heme side differed between the NOS, accounting for eNOS being the only isoform releasing NO at low nitrite concentrations. In human dermal microvascular endothelial cells in the presence of 10 μM extracellular nitrite under careful control of oxygen tension, the rates of NO formation determined by chemiluminescence were enhanced ~3.6-fold and ~8.3-fold under hypoxia (2 ppm O) and anoxia (argon) respectively compared to normoxia (~22 ppm O_2). NOS inhibitors inhibited this hypoxic NO release. Our data show that eNOS is unique in that it releases NO under all oxygen levels from normoxia to complete anoxia at physiological μM nitrite concentrations. The magnitude of the hypoxic NO release by the endothelial cells suggest that the endothelium could provide an appropriate response to acute episodic ischemia and may explain the observed eNOS-expression specific protective effect as a short-term response in animal models of acute hypoxia.

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YSF–64
Evidence for expression of human apolipoprotein A-I gene in monocyte/macrophage cells

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Apolipoprotein A-I (apo A-I) is a major protein component of high density lipoproteins in human, which take part in the reverse cholesterol transport from peripheral tissues to the liver. There are data that apo A-I demonstrates antiatherogenic and anti-inflammatory activities by influencing on monocyte/macrophage cells. In spite of this it is little known about local production of apo A-I in atherogenic lesions and expression of apo A-I gene in immune cells. Here we have investigated the regulation of apo A-I gene expression in THP-1 cells, a human monocyte/macrophage cell line, under the influence of tumor necrosis factor alpha (TNF alpha). It was shown for the first time that moderate expression of endogenous apo A-I gene occurs in THP-1 cells on both mRNA and protein levels. Quantitative RT-PCR demonstrates that the level of apo A-I mRNA was increased by 5.4 ± 1.1 fold over the control level in THP-1cells 24 hours after TNF alpha administration (10 ng/ml). TNF alpha also increases the content of intracellular apo A-I protein in these cells. PMA-induced differentiation of monocyte THP-1 cells into macrophages leads to increasing the basal level of apo A-I mRNA by 4.2 ± 0.3 fold over the level of monocytes but reduces the degree of TNF alpha-induced activation of apo A-I gene expression. Using specific inhibitors we have demonstrated functional roles of JNK and MEK1/2 in monocytes and NF-kappaB, JNK and p38 in macrophages in TNF alpha-mediated activation of apo A-I gene expression. Using agonist/antagonist approach we have shown that LXR is a positive and PPAR alpha is a negative regulator of apo A-I gene expression in THP-1 cells and both of those nuclear receptors appear to be involved in TNF alpha-mediated activation of apo A-I gene expression. Interestingly, the level of LXR alpha/beta is increased whereas the level of PPAR alpha is decreased in macrophages as compared with undifferentiated THP-1 cells, which seem to be cause of the elevation of basal apo A-I mRNA level in macrophages. These data show for the first time the expression of apo A-I on both mRNA and protein levels and the role of TNF alpha in activation of apo A-I gene expression in monocyte/macrophage cells. Our results suggest the possibility that apo A-I may be produced by monocyte/macropage cells in atherogenic lesions under inflammatory conditions during atherogenesis.

YSF–65
The Alternanthera mosaic virus coat proteins are able to form virus-like particles without RNA

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A new isolate of the Alternanthera mosaic potexvirus (AltMV-Ru), was purified from infected Portulaca grandiflora leaves. The EM and AFM images of purified AltMV-Ru particles were obtained. Flexible filamentous virions of AltMV-Ru are tipically for the Flexiviridae family (group of Potexvirus). Based on EM images, the average length and diameter of those virions are 550–600 and 12–13 nm respectively. Based on AFM images, the virions average height was ~6–8 nm. AltMV-Ru RNA and AltMV-Ru coat protein (CP) have been isolated, purified and characterized. For the first time it was shown that the AltMV-Ru CP subunits were able to form the virus-like particles (VLPs) with helical structure without AltMV-Ru RNA *in vitro*. The images of AltMV-Ru VLPs by EM and AFM under different pH-conditions were revealed. The length of AltMV VLPs is ranging from 60 to 2000 nm under pH 8.0. These particles aggregate in long beam-like structures to 10 μm under pH 4.0. Like other potexvirus (potato virus X) the encapsidated AltMV-Ru RNA is non-translatable *in vitro* (Atabekov *et al.*, 2000)

YSF–66
Nucleo-cytoplasmic export of Mouse mammary tumour virus RNAs

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The betaretrovirus Mouse mammary tumour virus (MMTV) replicates its RNA genome via a DNA intermediate integrated into the host genome, lika all other retroviruses. MMTV was recently shown to express a functional homologue of HIV-1 Rev – Rem (or regulator of expression of MMTV). Rem, by analogy to Rev, is believed to bind to its responsive element (RmRE) thereby mediating the nucleo-cytoplasmic transport of viral RNAs. Based on previous analyses, the MMTV RmRE was supposed to be located close to the 3' end of the genomic viral RNA. In order to more precisely locate the RmRE and to determine its proposed function, a series of MMTV molecular clones were constructed lacking different parts of the respective region. After transfection into MMTV permissive CrFK cells, viral RNA export was monitored via Northern blotting. By this means, a 400 nt long sequence spanning the Env-U3 region was identified to be essential for export of unspliced MMTV RNA. These results were confirmed in a second heterologous assay showing functional interaction of Rem and RmRE. Detailed evaluation of the obtained results indicated that single spliced viral RNA appeared to be exported Rem independently. This suggests that MMTV exploits different RNA export pathways for transport of its non-spliced and single spliced RNA species, respectively.

YSF–67
Metabolism of antitumor 9-amino-1-nitroacridine derivatives in HepG2 cells and its influence on cytochrome P450 enzymes

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The efficacy of anticancer therapy is limited by an inability to predict patient outcomes: tumour response and toxicity. This is complicated by the narrow therapeutic window and the fact, that many adverse drug reactions are caused by the cytochrome P450 dependent activation of drugs into reactive metabolites. Antitumor 9-amino-1-nitroacridine derivatives, C-857 and C-1748, belong to a new set of antitumor compounds developed in our laboratory. One of them, C-1748 (Capridine-beta) which expressed low toxicity in animals and potent antitumor activity againts prostate cancer was selected to preclinical studies. We showed earlier that the metabolic activation of 1-nitroacridines is necessary for activity of these compounds towards tumor cells, however C-1748 was shown to be less reactive than C-857 with human and rat liver microsomes and with human *E. coli* recombinant CYPs. We hypothesized that slight metabolism of C-1748 is a probably reason of its reduced toxicity in animals. In recent work we showed that studied compounds penetrated into human hepatoma HepG2 cells and were metabolized to numerous diverse and reactive products. The present work aimed to evaluate whether the studied compounds influence the level of cytochrome P450 enzymes with reference to kinetics of their metabolism. Microsomal fraction from HepG2 cells were prepared and analyzed by Western blotting for members of the CYP1, CYP2, CYP3 subfamilies. The highest protein level was observed in the case of CYP3A4 what indicated that C-857 and C-1748 underwent metabolic transfromations mainly with this izoenzyme. In the next step, HepG2 cells were incubated with specified concentration of C-857 and C-1748 and then HPLC analysis of metabolites as well as Western blot analysis of CYP3A4 level in HepG2 cell were performed after identical periods of time. The obtained results showed that while C-857 induced a strong reduction of CYP3A4 protein level after 6 hours of incubation, C-1748 caused only slight reduction of this protein after all time of incubation. Correspondingly, the highest concentration of C-857 metabolites in HepG2 was found after 3 hours, whereas the concentration of C-1748 metabolic product was stable till 24 hours. Therefore, the formation of reactive metabolites and reduction of CYP3A4 protein level was observed simultaneously. In conclusion, we have found that the formation of reactive metabolites of studied compounds is associated with the downregulation of CYP3A4.

YSF–68
Cathepsin K exists in two functionally distinct conformations at neutral pH

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Cathepsin K is a major player in extracellular proteolysis, performing key roles in bone resorption and numerous other proteolytic processes. In this work we investigated unconventional

mechanisms of extracellular cathepsin K regulation that occur through interactions of modifiers with enzyme sites remote from the active center. At neutral pH, the enzyme exists in two conformational states that differ in their kinetic properties and susceptibility towards active site-directed inhibitors. The conformational equilibrium is sensitive to ionic strength, enzyme and substrate concentration. Some abundantly occurring extracellular macromolecules modify the equilibrium between the 'open' and the 'closed' states. Glycosaminoglycans promote the 'open' conformation of cathepsin K and act as nonessential activators. Heparin is a much more potent activator than chondroitin or dermatan sulfate in assays with both low molecular mass substrates and polymeric elastin. Kinetic measurements show that chondroitin/dermatan sulfate bind the enzyme at one site, while heparin binds at two sites and exhibits a unique stabilizing effect on cathepsin K. Two abundantly occurring matrix proteins, clusterin and vitronectin, were found to influence the conformational flexibility of cathepsin K and to stimulate its elastinolytic activity. Both proteins compete with glycosaminoglycans for binding the enzyme, indicating that they share the same binding site. This was confirmed by cross-linking experiments, in which the binding moiety of vitronectin was mapped to the N-terminal somatomedin B domain. Taken together, our work presents the first in-depth investigation of the conformational flexibility in cysteine cathepsins and offers novel insights into the mechanisms of cathepsin regulation in the extracellular space.

YSF–69
Role of the PI3K/Akt/FoxO pathway in the pathogenesis of mantle cell lymphoma cells

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Background: The PI3K/AKT signalling pathway controls many biological functions including cell proliferation, survival and insulin responses. This pathway is essential for proliferation of haematopoietic cells and for immune homeostasis, and is involved in the development of haematological neoplasia. FoxO transcription factors are negatively regulated by the PI3K/Akt pathway through phosphorylation. When active, FoxOs induce cell cycle arrest and apoptosis, acting as potent tumour suppressors. Although recent genetic evidence points towards a central role of FoxO3a in lymphomagenesis, little is known regarding the role of FoxO factors in MCL. Mantle cell lymphoma (MCL) is a subtype of B-cell non-Hodgkin lymphoma characterized by the t(11;14) chromosomal translocation, which results in aberrant overexpression of cyclin D1. This genetic event is present in virtually all cases of MCL, but it is not sufficient by itself to cause lymphoma, which means that elucidation of additional genetic lesions is essential and will provide insights towards a specific therapy.
Objectives: (1) to study the role of FoxO3a in transformation and cell survival; (2) to evaluate the clinical relevance of FoxO3a in cancer drug therapy, and its role as a functional target of new therapeutic agents.
Results: We have evaluated the relative role of the PI3K/AKT/FoxO3a pathway in a panel of MCL cell lines corresponding to different stages of disease progression. The cellular response to various chemical inhibitors of the pathway such as LY294002, Rapamycin and Triciribine has been analysed. Rapamycin, which does not affect FoxO3a activity, has little effect on cell viability, whereas response to LY294002 or Triciribine differs among the various cell lines. In Jeko-1 cells treatment with inhibitors decreases AKT and FoxO3a phosphorylation and increases expression of target genes, leading to cell cycle arrest and cell death. Interestingly, in LY294002-resistant JVM-2 cells FoxO3a protein levels are undetectable by Western blot, suggesting an important role for FoxO3a in LY294002-mediated cellular responses. We have also used the inhibitor of FoxO nuclear export Psammaplysene A and will present data regarding its effect on MCL cell survival. The conventional chemotherapeutic drugs Doxorubicin and Oxaliplatin have also been tested alone and in combination with PI3K/AKT/FoxO3a inhibitors.
Conclusion: We have described the role of FoxO3a in the growth and survival of MCL cells and we have tested its clinical relevance as a target of different therapeutic agents.

YSF–70
Proteomic analysis of Paramecium Rab7 GTPases encoded by paralogous genes

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Background and objectives: Identification of protein products encoded by paralogous genes is a challenging task due to a high sequence homology of these proteins. Such an example is the genome of the unicellular eukaryote model cell Paramecium in which four whole-genome duplication occurred (Aury *et al.* 2006). We identified two genes encoding Rab7 GTPases of 206 deduced amino acids that are 97.6% identical and differ in their C-termini with four amino acids (Surmacz *et al.* 2006).
Methods: SDS–PAGE of Paramecium cell fractions was followed by either silver/Brilliant Blue G staining or Western blot with the specific antipeptide antibodies (Abs) generated against C-termini of Rab7a and Rab7b, respectively. The stained protein bands corresponding to those cross-reacting with Abs were cut off and analyzed by Liquid chromatography–tandem mass spectrometry (LC–MS/MS). Protein identification was made using MASCOT ver. 1.9 combined with extensive search of sequence databases. Michigan Conventional approach to proteomics involves cleaveage of the studied proteins into smaller peptides with trypsin as a protease. LC–MS/MS revealed the presence of the peptides matching both the Rab7 isotypes such as ^{158}KDATNIEQAFQDIAKA^{173}, ^{80}GADCCVLVYDITNPK^{94} (containing RabF5 motif) and ^{39}ATVGADFMAK^{48} (including RabF1). Mapping of proteolytic sites in Rab7a and Rab7b cleaved by different endopeptidases revealed that trypsin digestion is inappriopriate to distinguish these proteins since a number of very short peptides and even the single amino acid residues were formed. To solve this problem Asp-N endopeptidase was used to digest the excised gel bands. In mass spectrometry following peptides were obtained: ^{169}DIAKAAASQEK^{179}, ^{15}GDSGVGKTSLMNQYVNARFTQQYRATVGAD^{44} (containing RabSF2 motif), ^{180}DEEIFFPTTVTVTKQ^{194} and ^{62}WDTAGQERFQSLGGAFYRGADCCVLVYD^{89} (including RabF3 and RabF4 motifs) – present in the both isotypes of Rab7 and ^{180}DEEIFFPTTVTVTKQSQKPQAKQGGCC^{206} that is characteristic for Rab7a only. This peptide contains all the four distinct amino acid residues not present in Rab7b. Moreover, identification of this peptide in the band of M_r˜22.5 kDa is consistent with results of Western blot since Rab7b is migrating at M_r∼25 kDa.
Conclusions: Proteomic analysis identified the peptides fully consistent with the deduced sequences of Rab7 isotypes that include characteristic Rab family (RabF) and subfamily (RabSF) motifs. Overlapping peptides were obtained after cleavage with two proteases prior to MS. Only unconventional cleveage with Asp-N endopeptidase enabled distinguishing of Rab7a from Rab7b, the products encoded by paralogous genes.

YSF–71
Regulation of neuronal migration by proneural GTP-binding protein pathways

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After their birth, neurons usually undergo radial or tangential migration to reach defined locations where they integrate into functional circuits. Recent data demonstrated that proneural factors such as Neurogenin (Ngn) 1, Ngn2 and Mash1 promote cell migration when they are overexpressed in neural progenitors (Ge *et al.*, 2006). Rnd2 has been found to mediate Ngn2 activity in cell migration in the cerebral cortex (Heng *et al.*, 2008). However, the mechanisms by which Mash1 activates neuronal migration appear to be different and are still unknown. By screening putative Mash1 targets identified in expression microarray experiments, we found genes that regulate cell migration in other systems like RhoE/Rnd3. As its role in the developing nervous system has not yet been addressed, we have begun to study the role of Rnd3 in the radial migration of cortical projection neurons. Rnd3 is expressed in the developing cortex from E12.5 to E16.5. Rnd3 knock-down in the dorsal telencephalon at E14.5 results in a radial migration defect of cortical projection neurons and increases the fraction of dividing progenitors by promoting cell cycle re-entry. These migration defects following Rnd3 knock-down are due to a distinct function of Rnd3 in post-mitotic neurons and they are more severe when Rnd2 is also knocked-down. Moreover Rnd3 knock-down affects the morphology of migrating neurons. Mash1 is required for Rnd3 expression in the dorsal telencephalon and several highly conserved Mash1 binding sites (known as E-boxes) are located in non-coding sequences of Rnd3 gene. Some of these E-boxes are able to bind Mash1 suggesting that this proneural factor directly regulates Rnd3 expression in telencephalic neurons. These results demonstrate that the Mash1-Rnd3 pathway plays a critical role in the migration of projection neurons in the developing telencephalon. The expression, regulation and knockdown phenotypes of Rnd2 and Rnd3 also indicate that the two genes are part of different regional programmes of neuronal migration.

YSF–72
Microarray analysis of differentially expressed genes in osteoblasts and osteocytes in murine calvarial tissue

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Background: Comprehensiveanalysis of gene expression patterns and regulatory networks of cells involved in skeletal development and remodeling is a prerequisite for complete understanding of physiological bone structure, function and homeostasis.
Objective: To determine the differential gene expression between osteoblast and osteocytes that characterise their true *in vivo* setting.
Methods: We have developeda dual GFP reporter mice in which osteocytes are expressing GFP(topaz) directed by the DMP-1 promoter, while osteoblasts are expressing of GFP(cyan) driven by 2.3 kb fragment of theCol1a1 promoter. To isolate distinct populations of cells from mouse neonatal calvarial tissue we utilized sequential enzymatic digestion followed by FACS analysis. Cells expressing GFP(topaz) were collected as osteocytes and cells expressing only GFP(cyan) were collected as osteoblasts. Cell suspensions were subjected to RNA extraction, *in vitro* transcription and labeling of cDNA. Gene expression was analyzed using Illumina mouse WG-6v1 BeadChip. Following normalization of raw data for all four biological replicas combined, 3444 genes were called present in all three sorted cell populations: GFP negative, Col2.3cyan$^+$ (osteoblasts), and DMP1topaz$^+$ (preosteocytes and osteocytes). Statistical analysis (SAM, LIMMA) resulted with overlapping list of 561 and 385 genes with significant changes in gene expression. We further analysed genes that exceeded 2-fold change for gene expression between DMP1topaz$^+$ and Col2.3cyan$^+$ cells. Selected genes were classified and grouped according to their associated gene ontology terms.
Results: Genes clustered toosteogenesis and skeletal development such as Bmp4, Bmp8a, Dmp1, Enpp1, Phexand Ank were highly expressed in DMP1topaz$^+$ cells. Most of the genes encoding extracellular matrix components and secreted proteins had lower expression in DMP1topaz$^+$ cells whilemost of the genes encoding plasma membrane proteins were increased.Interestingly a large number of genes associated with muscle development and function and with neuronal phenotype were increased in DMP1topaz$^+$ cells indicating some new aspects of the osteocyte biology.
Conclusion: Utilization of described approach in isolation of osteocyte and osteoblast cell populations and their subsequent microarray analysis allowed us to identify osteoblast and osteocyte gene expression profiles representing their true *in vivo* state. Furthermore it allowed us the identification of some new genes and pathways with potential role in bone biology.

YSF–73
Molecular mechanisms of chromophore modifications in fluorescent proteins

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Here we present our investigations on chromophore formation within novel fluorescent proteins derived from Anthozoa corals. Currently, fluorescent proteins (FPs) are indispensable tools for live cell imaging. In the past decade a number of FPs possessing novel properties nave been cloned from Anthozoa corals. Thus family of the GFP (green fluorescent protein from jellyfish Aequorea victoria) was extended appreciably. Some of the novel proteins have a significant spectral shift to the red region in comparison to GFP, allowing extensive multicolor labeling. Another group of proteins was able for photoactivation and photoinduced color transformation that opened facilities for precise photolabeling and tracking. Mostly, these features depend on the chromophore structure of these proteins. We investigated chromophore formation within several fluorescent proteins possessing significant red shift and found that their chromophores are the product of oxidation or oxidative decarboxylation of the GFP-chromophore. As a result the chromophore is extended with the acylimine group. Furthermore we investigated chromophore formation within kindling fluorescent protein asFP595 and showed that it is a product of acylimine hydrolysis, resulting in a keto-derivative of the GFP-chromophore. Also we investigated green-to red photoconversion of DendFP and showed that this phenomenon is conditioned by elimination reaction at the first

chromophore-forming histidine residue followed by GFP-chromophore extension with imidazolylethenyl group. Thus our findings suggest that GFP-chromophore extension is controlled by the reactions of acylimine formation and degradation, and by photoelimination reaction.

YSF–74
Structural mechanism of yeast aquaporin gating

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Aquaporins are transmembrane proteins which facilitate the flow of water through cellular membranes. An unusual characteristic of yeast aquaporins is that they frequently contain an extended N-terminus of unknown function. Here we present the X-ray structure of the yeast aquaporin Aqy1 from Pichia pastoris at 1.15 Å resolution. Our crystal structure reveals that the water channel is closed by the N-terminus, which arranges as a helical bundle, reminiscent of that seen for the mechanosensitive potassium channel MscL. Nevertheless, functional assays show that Aqy1 has appreciable water transport activity and aids survival during rapid freezing of P. pastoris. These findings establish that Aqy1 is a gated water channel. Mutational studies in combination with molecular dynamics simulations imply that this property may be conferred by a combination of regulation by phosphorylation and mechanosensitive gating.

YSF–75
RNA editing as an epigenetic mechanism contributing to the tumor phenotype

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RNA editing is a post-transcriptional modification, altering the sequence of RNA from that encoded in the DNA. Adenosine-to-inosine RNA editing is an essential site-specific modification, catalyzed by the ADAR family. It was considered a rare modification; however, we and others have shown that it is actually widespread affecting thousands of genes in millions of sites in the human transcriptome, mainly in Alu-sequences. We studied the effect of this modification in cancer and identified significant global hypo-editing of Alu sequences in brain, prostate, lung, kidney, and testis tumors. This finding is best illustrated by the analysis of brain tumors compared to normal brain tissues where editing levels are significantly reduced as shown both bioinformatically ($P < 10^{-58}$) and experimentally ($P < 10^{-42}$). Comparison of editing levels in coding-sequences revealed significant alterations in brain, oral and lung tumors compared to normal tissues. Hence, different mechanisms control behavior of the two classes of editing sites in cancer. While general under-editing in cancer tissues was identified in repetitive elements, site-specific editing seems to function in a gene-specific manner possibly depending on the advantages provided by its product. Analysis of the RNA-editing enzymes reveals decreased levels of expression in tumors, correlating with the reduced global editing level. Overexpression of ADAR1/2 in tumor cell lines resulted in altered editing levels and decreased proliferation rate. Finally modulation of the editing enzymes in various tumors was shown to affect the expression level of specific microRNAs. Our work suggests a novel type of epigenetic control in cancer which may have major implications in the understanding and diagnosis of cancer.

YSF–76
The cannabinoid WIN induces DR5 receptor expression in hepatoma cells sensitizing them to TRAIL signal

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The tumor necrosis factor-related apoptosis inducing ligand (TRAIL), a member of TNF superfamily, is of special interest for cancer therapy since appears to specifically kill a wide variety of cancer cells while sparing most normal cells. However, many malignant cells are resistant to TRAIL and therapeutic strategy are searched to sensitize cancer cells to TRAIL-induced apoptosis. It has been demonstrated that cannabinoids, the active constituents of Cannabis sativa, induce growth inhibition and apoptosis in different tumor cell lines. Recently, we have demonstrated that sub-optimal doses of WIN sensitize HepG2 cells to TRAIL and WIN/TRAIL combination (5 mM WIN/20 ng/ml TRAIL for 24 hours) results in a strong synergistic cytotoxicity, revealed by combination indices < 1. The aim of this study was to elucidate the mechanism through which WIN regulates TRAIL signal. We found that the incubation with WIN resulted in the decrease of TRAIL decoy receptor DcR2 and the increase in both the transcript and protein expression of DR5, one of the death receptors bound by TRAIL. It has been documented that DR5 up-regulation can be mediated by the binding of the transcription factors PPARg, AP1 or CHOP into DR5 promoter. Although in HepG2 cells WIN-dependent DR5 up-regulation was accompanied by PPARg increase, no significant decrease in DR5 expression was observed after incubation with the PPARg antagonist GW9662 or in the presence of PPARg siRNA, indicating that WIN modulates the expression of DR5 through a PPARg-independent mechanism. However, the addition of GW9662 significantly counteracted the reduction in cell viability and in the levels of Akt, cFlip, and IAP-2 protein induced by combined treatment, suggesting an involvement of PPARg in the apoptotic pathway. Moreover, DR5 up-regulation is not dependent on JNK because no significant difference was observed in the phosphorylated active form of the enzyme after incubation with WIN. Instead, we observe an increase in the level of the stress-regulated protein p8, an essential mediator of cannabinoid antitumoral action and an activator of CHOP gene, after incubation for short times with WIN. In summary, we hypothesize that sensitization of hepatoma cells to TRAIL signal by WIN is a consequence of DR5 up-regulation induced by the cannabinoid through the increase in the level of the transcription factor CHOP mediated by p8.

YSF–77
Identification of the third sodium site in the neuronal glycine transporter GLYT2

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Glycine neurotransmitter transporters (GLYTs) control the availability of glycine at the glycinergic synapses. GLYT1 is the main regulator of the glycine levels in the synaptic cleft and catalizes sodium/chloride/glycine cotransport with a 2:1:1 stoichiometry. The neuronal isoform, GLYT2 that supplies glycine to the presynaptic terminal, transports an additional sodium ion displaying a 3:1:1 stoichiometry. We have generated, and experimentally validated, a model of the three-dimensional structure of GLYTs by homology modelling with a procaryotic orthologue (LeuTAa). Using molecular dynamics simulations coupled to electrostatic calculations of the transporters in the presence of sodium, we have identified the residues involved in the additional sodium site of GLYT2 (Na3), and experimentally confirmed its use in this transporter but not in GLYT1. The replacement of Asp471 located in TM6 of GLYT2, but not the equivalent position in GLYT1 (Asp295), reduced sodium affinity and sodium cooperativity of glycine transport. Cysteine substitution of the two aspartates yielded GLYTs differentially sensitive to methanethiosulfonate (MTS) reagents in a sodium and glycine-dependent manner. An efficient allosteric communication between Asp471 and the Na1-2 sites was inferred from the differential responses to positively charged MTS reagents by D471C (GLYT2) and D295C (GLYT1). The prevention of sodium binding to Na3 by biotin-coupled MTS permitted the isolation of a D471C intermediate of the transport cycle not detected for D295C. D471C, but not D295C, was sensitive to the charge of the MTS reagent. Sodium protected D471C from inhibition with negatively charged MTS at a conformationally restricted temperature, indicating direct binding to position 471.

YSF–78
Stress response of horseradish to toxic metals

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Most organisms have countered exposure to toxic substances with the development of detoxification systems to transform, metabolize and eliminate such compound from tissues. Many toxic elements are taken up by plants along with essential elements. Knowledge of the genetics and biochemical processes involved in metal uptake is essential for development of transgenic plants with improved phytoremediation capability. In plant metabolism, glutathione S-transeferase and peroxidase activities have a direct relation to the effect of stress. Changes in the activites of the enzymes were therefore studied. We deal with toxic metals uptake in horseradish. We also concerned to factors that have an influence on accumulation and distribution of metal ions in plant parts. A hairy root culture of horseradish (*Armoracia rusticana* L.) was treated by U(VI), Ni(II) or Cd(II) ions or their combinations. Other experiment was focused to different additions in hydroponic solution. Plants were grown in Hoagland solution with U(VI), Ni(II) or Cd(II) ions or their combinations. Each solution was treated by chelating agents (EDTA, citric acid, tartaric acid or oxalic acid) or humic acids. The analysis of ions contents in plant parts showed that ions are deposited mainly in roots. The absence of phosphates in solution increased amount of uranium in plants. This can be cause by better availability of uranyl ions from solution. The accumulation of cadmium and nickel in hairy root culture increased in time and apparently cadmium uptake was affected by uranyl ions. On the other hand uranium ions were accumulated within few hours and their amount did not increase during the cultivation time. Uranyl and cadmium ions caused increase of peroxidase activity but nickel do not. GST activities of culture cultivated with cadmium or nickel were increasing but uranyl ions had minimal influence on both enzymes. It seems that uranium have a different uptake mechanism than cadmium or nickel.

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YSF–79
Evaluation of blood antioxidant status and lipid peroxidation in pediatric acute lymphoblastic leukemia and non-Hodgkin lymphoma

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Background: Reactive oxygen species (ROS) are an inevitable result in cells that use oxygen for energy production. ROS are known to play a dual role in biological systems, being either beneficial or harmful to living organisms. At high levels, ROS can be mediators of damage to DNA, proteins and lipids, leading to cellular dysfunction and cell death. It is well recognized that oxidants play a role in several stages of carcinogenesis, but information about the activities of antioxidant enzymes and oxidative stress markers are rare and conflicting in children with various haematological malignancies.
Objectives: We evaluated the antioxidant systems and lipid peroxides as markers of the oxidative status in children with acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL).
Methods: The activity of antioxidant enzymes (glutathione peroxidase-GPx, superoxide dismutase-SOD and catalase-CAT) in erythrocytes and lipid peroxides level estimated as thiobarbituric acid reactive substances (TBARS) in plasma of 10 ALL children and 6 NHL children was compared with those from healthy controls. The samples were taken during therapy, before the next treatment, to determine the accumulative oxidative stress. Data are expressed as mean-standard deviation.
Results: Children from the two groups had lower activity of GPx and CAT than controls. GPx activity had lower values in NHL group (621.08 ± 18.23 U/l, versus 818.76 ± 361.72 U/l). CAT activity had the lowest value in blood from ALL children (62.86 ± 5.85 k/gHb, versus 75.88 ± 20.42 k/gHb). There were no apparent differences in SOD activity in children suffering from ALL and NHL, SOD activity having a value at the lowest limit of the normal range (196.3 ± 8.77 U/ml in ALL children, 194.9 ± 13.17 U/ml in NHL children). Plasma TBARS had a higher value in NHL than in ALL children (3.848 ± 0.431 versus 3.148 ± 0.319 nmole/ml).
Conclusions: In children from both groups we noted an impaired antioxidant status. Findings may indicate a link

between decreased antioxidants and increased levels of cell alterations due to oxidative damage, supporting the idea that there is a persistence of oxidative stress in these haematological malignancies. The difference noted between the two groups could be associated with the protocol for treatment. For this reason we intend to continue the study in children just diagnosed compared to different phases of treatment, remission induction and remission maintenance.

YSF–80
The role of calcium ions in lipid bodies life-cycle of marine bacterium Alcanivorax borkumensis

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Alcanivorax borkumensis is an ubiquitous marine *hydrocarbonoclastic bacterium* with ability to degrade an exceptionally broad range of alkane hydrocarbons but only few other substrates. Its lipid metabolism is characterised by production of three lipid groups (triacylglycerols, polyhydroxyalkonates and free fatty acids) that are deposited inside the cell in the form of lipid bodies. Its metabolism makes *A. borkumensis* an interesting tool for several biotechnological applications including production of biodegradable plastics and bioremediation of petroleum oil contamination in marine ecosystems. Ca^{2+} play an important role in the life-cycle of lipid bodies in several organisms (e.g. mammalian and plants), mainly during biogenesis and mobilisation phase. For that reason the evidence of calcium presence on the surface of lipid bodies of *A. borkumensis* evoked the question of potential calcium role in their life-cycle. We use several approaches, such as protein profile determination and lipid analysis, to elucidate this role. Our results showed that Ca^{2+} are not essential for growth of *A. borkumensis* but influence its progress. Ca^{2+} probably enhance lipid accumulation at low concentration of carbon source and they are probably as well connected with the mobilisation of lipid bodies, especially in the starvation period. Contrary, our results denied the theory of calcium detoxification by its location on the surface of lipid bodies as the mutant deficient in lipid bodies formation was able to growth in the carbon-rich medium.

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YSF–81
Energetic metabolism of myelinated axons: a new trophic role for myelin sheath

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Background: Neuronal functioning requires a lot of energy. Brain consumes more than 20% of the oxygen and glucose of the whole organism. This is quite surprising because brain mitochondrial density is lower than in other organs with less energy demand. Also, myelin sheath, the multilayered membrane allowing the nerve to transmit its impulses rapidly, exerts an as yet unexplained neuro-trophic role. In fact, in demyelinising diseases, like in Multiple Sclerosis, a lowering of conduction speed but also an axonal necrosis, is observed.
Objectives: Aiming at contributing to the understanding of the causes of the axonal degeneration consequent to myelin loss, our principal objective was to demonstrate that the electron transport chain (ETC) is functional in myelin, to carry out oxidative phosphorylation, for ATP supply to the axon.
Methods: Experiments were conducted on isolated myelin vesicles (IMV), obtained according to the method of Norton and Poduslo. Both an imaging and a biochemical approach were utilized. Transmission Electron and confocal microscopy as well as oxymetric, fluorimetric, luminometric and Semiquantitative Western Blotting (WB) analyses were performed.
Results: We observed that IMV: (i) are able to consume oxygen with NADH and Succinate as respiring substrates (ii) display a proton gradient across their surface; (iii) contain F_0–F_1 ATP Synthase and the ETC complexes, which are catalytically active. Mitochondrial contamination, as assessed by semiquantitative WB with antibodies against proteins typical of the mitochondria, was found to be negligible in IMV.
Conclusion/Application to practice: Data suggest that the whole redox chain is present in myelin sheath and that it is catalytically active in aerobic ATP production, which may be pivotal to the high energy demands of axons. This basic study will shed light on the aetiophatogenesis of many demyelinising diseases.

YSF–82
Molecular biosensor for *in vitro* cAMP measurements

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Cyclic adenosine monophosphate (cAMP) is an important second messenger involved in neural signal transmission. The level of cAMP reflects the state of certain enzymes and other proteins found inside the cell or in the cell membrane, including seven trans-membrane receptors. Measuring level of cAMP can therefore give us valuable information about receptor functionality. Currently, immunological detection is the method of choice for measuring cAMP *in vitro*. During the recent years less labour-intensive and more flexible fluorescence methods have been developed. The most approved methods utilize certain cellular proteins as sensor molecules for detection of cAMP both *in vivo* and *in vitro*. Protein kinase A (PKA) and Epac are proteins specifically binding cAMP in various cell types. Nikolaev *et al.* [JBC (2004), 279, 34215] have developed a molecular biosensor, a genetically modified Epac protein containing a pair of fluorescence proteins, CFP and YFP. The monomolecular structure and ease of production of the Epac sensor protein have made it our tool of choice in cAMP measurements. Our aim is to apply the Epac-cAMP biosensor in a form of a purified protein for measuring cAMP level in various biological samples including tissue homogenates to determine dopamine D_1 and adenosine A_{2A} receptor activation. Therefore Epac-cAMP sensor protein was tagged with a 8 amino acid long Streptag-sequence, which allows highly selective purification of the protein from a crude cell lysate by using commercially available Streptactin affinity columns. For expression of the sensor protein in Sf9 cells we use baculovirus expression vector system which is cost-effective and allows fast production of the protein of interest with all the necessary folding and post-translational modifications

YSF–83
Rationally designing new analgesic peptides: from biophysical screening to *in vivo* proof-of-concept

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Kyotorphin was first discovered in 1979 and reported as an endogenous analgesic agent in the brain. Attempts to utilize it as an analgesic have, however, been unsuccessful due to its inability to cross the Blood–Brain-Barrier (BBB). A library of kyotorphin derivatives was design and synthesized to overcome this problem. Biophysical studies were carried out using fluorescence methodologies to characterize the peptides interaction with lipid-membrane model systems. Partition coefficient quantification showed a clear preference of the derivatives towards zwitterionic and anionic fluid lipid bilayers under physiological conditions, mimicking mammalian membranes. *In vivo* studies with systemic administration of the peptides were performed to evaluate the anti-nociception behavior in experimental models of acute, tonic, chronic and neuropathic pain. Unlike kyotorphin, only active when injected into CNS, a subset of the compounds displayed remarkable analgesic properties from doses as low as 0.85 mg/100 g, indicating the capacity to cross the BBB. An observed correlation between higher partition/insertion in the membrane and enhanced analgesic action provided further insight into the mechanism of these compounds and proved this strategy valuable for early screening in CNS drug development. Additionally, citotoxicity was assessed in V79 lung fibroblasts. Up to 100 mM, none of the compounds affected either the metabolic activity in the mitochondria or the membrane integrity. With a clear affinity to mammalian membranes, demonstrated *in vivo* by the analgesic effect after i.p. injection, these new kyotorphin derivatives display the ability to cross the BBB. This, together with the lack of toxicity, makes them molecules with promising pharmacological applications.

YSF–84
Development of a green fluorescent reporter for the bio-assay of anti-cancer drugs with anti-oxidant activity

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Background: Reactive oxygen species (ROS) cause cell damages through protein oxidation leading to pathological processes like cardiovascular disease neurological degeneration and cancer. Antioxidant molecules, due to their ability to scavenge and neutralize free radicals, might play a pivotal role in the prevention of these disease. Interestingly, besides neutralizing ROS, it's now proved that promising antioxidant molecules, including green tea polyphenols (GTPs) quercetin and sulphoraphane, induce the expression of genes involved in the cellular protection against oxidative stress.
Objectives: Regulation of these detoxifying genes expression by antioxidant chemopreventive compounds is mediated by the electrophile-responsive element (EpRE), a *cis*-acting regulatory sequence involved in the coordinated transcriptional activation of genes associated with phase II biotransformation. To this extent we constructed a new expression vector, cloning the minimal promoter of NADPH quinone oxydoreductase-1 (NADPH(Q)1), comprising the EpRE element, upstream the Zoanthus sp, a gene codifyng a green fluorescent protein, in a promoterless, commercially available, expression vector (pZSGreen1-1).
Matherials and Methods: When transiently transfected this expression vector acts as a molecular probe, reporting the state of activation of NADP(Q)1, in living cells in response to natural or synthetic antioxidant substances.
Results: Preliminary results in immortalized and transformed prostate epithelial cells indicate that the reporter is activated by tert-buthylhydroquinone the standard inducer of EpRE controlled genes, and also by GTPs, well known natural chemopreventive molecules.
Conclusions: Therefore this new molecular probe might be employed for the screening of novel chemopreventive substances acting through the activation of phase II detoxifying enzymes.

YSF–85
Erythropoietin protect against cisplatin induced apoptosis and oxidative damage in rat kidney

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Background: Cisplatin is an active cytotoxic agent that has proved to be successful in the treatment of various types of solid tumors. The drug-induced nephrotoxicity has been very well documented in clinical oncology. Erythropoietin (EPO), a renal cytokine regulating haematopoiesis, has recently been shown to exert important cytoprotective and antiapoptotic effects on several experimental injuries. The aim of this study was to explore whether the EPO protects against cisplatin-induced nephrotoxicity and oxidative damage *in vivo*.
Methods: Adult male Wistar rats (120–140 g) were divided into six groups of six animals each. The control group (group 1) was treated only with an intraperitoneal injection of NaCl 0.9%; animals in group 2 received only EPO and the third group received only cisplatin. EPO and cisplatin were simultaneously administrated to animals of the group 4. Five days of cisplatin treatment was performed following administration of EPO for the animals in group 5 and a 5 days of cisplatine pre-treatment was applied to the animals in group 6 before administration of EPO. Cisplatin (6 mg/kg) and EPO (3000 UI/kg) were intraperitoneally injected as a single dose. Biochemical and histopathological methods were used for the evaluation of the nephrotoxicity. The concentrations of serum creatinine and malondialdehyde (MDA) were determined in plasma. The levels of pro/antiapoptotic proteins and the caspase-3 enzyme activitiy level were determined in kidney tissue.
Results: Administration of cisplatin to rats induced a marked renal failure, characterized by a significant increase in plasma creatinine and MDA concentrations and by a high level of tubular apoptosis. EPO administration produced amelioration in biochemical indices of nephrotoxicity in both plasma and kidney tissues when compared to group 3. EPO present a direct effect against cisplatin induced apoptosis in kidney by the upregulation of antiapoptotic proteins expression: Bcl-2, Hsp70 and Hsp27; and by the downregulation of proapoptotic proteins expression: Bax and P53. Caspase-3 enzyme activity level was significantly reduced in animals treated with EPO.
Conclusions: Results from this study suggest that, in addition to its well-known erythropoietic effects, EPO inhibits apoptotic cell death and protect kidneys against cisplatin-induced apoptosis and oxidative stress in rat, particularly when it was administrated 24 hours before cisplatin treatment.

YSF–86
Multidrug resistance and apoptosis in human leukaemia cells

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Background: Leukaemia cells are undifferentiated cells which develop uncontrolled proliferation. The differentiating therapy sometimes leads to drug resistance. Multidrug resistant phenotype (MDR) is a very common phenomenon that appears in tumor cells making them resistant to chemotherapy.
Objectives: We are specially interested in identify genes potentially involved in apoptosis resistance in leukaemia with MDR phenotype. We want to use these genes as possible targets to increase therapeutical success in chemoresistant tumors.
Methods and Results: In order to generate resistant sublines with acquired MDR phenotype we treated leukaemia cells (HL60 and K562) to increasing doses of daunomycin. Resistant leukemia cell sublines were less sensitive to apoptosis than their respective parental counterparts. To determine whether there is a relationship between differentiation and MDR phenotype, these cells became differentiated by adding 12-*O*-tetradecanoilforbol-13-acetate (TPA). Flow cytometry analysis showed that parental cells reached this stage, but resistant sublines did not. Interestingly, we observed that parental mature cells became resistant to the apoptosis inducing agent etoposide. This result suggests that differentiated cells develop changes that make them resistant to apoptosis like resistant cells do.We studied RNA expression profiles using microarray technology and observed substantial changes between resistant and parental phenotype. From this study, we found upregulation of some genes in mature parental cells, which were upregulated in resistant non-differentiated cells as well.
Conclusion: Differential gene expression studies can help us to identify genes involved in the acquisition of MDR phenotype which can be in the future targets for a successful chemotherapy.

YSF–87
Dendritic cell number in prognosis of respiratory papillomatosis outcome

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Respiratory papillomatosis associated with human papilloma virus (HPV) infection is the most common benign laryngeal neoplasm. Dendritic cells (DC) play a major role in antitumor immune response and their functional state can determine the outcome of HPV infection. Up to now common superficial marker of DC–CD1a – was used for immunohistological studies of laryngeal papillomas and the evaluation of matured DC density was not conducted. The aim of our study was to estimate the density both of immature and mature DC in laryngeal papillomas associated with HPV types 6/11 infection, using immunolabeling of dendritic cells with anti-CD 1a (common marker for DC) and anti-CD 83 (marker of DC maturation) antibodies. Our study included 40 randomly selected biopsy specimens from patients with HPV positive laryngeal papillomatosis aged from 1 to 20 year. DC were immunohistochemically labelled with anti-CD1a and anti-CD83 antibodies. The density of DC was analysed in epithelial layer and lamina propria. Statistical analysis was carried out using the software TANAGRA 1.4.21. All data are presented as median with 25%–75% quartiles. Based on clinical data we divided our sample of patients into 2 groups according to severity of disease. For subgroup of patients with high number of operations (more than 3), early disease onset (under 3 years of age) and lingering disease duration (more than 1 year) we found a statistically significant increase of $CD83^+$ DC in the epithelial layer, in comparison with the control and with the second group of patients. We did not notice any alterations of $CD1a^+$ DC distribution in larynx papillomas for two groups of patients. $CD1a^+$ cell count was a little higher in the epithelium of patients with papillomatosis compared to the control samples, but this difference was insignificant. Thus, we observed the correlation between the papillomatosis severity and $CD83^+$ DC count in the epithelial layer of laryngeal papillomas. Our results indicate that DC in laryngeal papillomas retained their capacity to be activated by HPV antigens. However, the processes observed in the group of patients at high risk for recurrent respiratory papillomatosis can be an evidence of the impairment of DC migration with their retention within the epithelial layer. So the increased number of $CD83^+$ DC in the epithelial layer of papillomas can be a valuable prognostic factor for the severe course of disease.

YSF–88
DsbE-like disulfide oxidoreductases from Neisseria meningitidis: Biochemical and functional characterization

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DsbEs belong to a family of typical membrane-anchored periplasmic thiol/disulfide oxidoreductases that exhibit specific reducing activities in a highly oxidizing environment. DsbEs are essential for c-type cytochrome maturation. *Neisseria meningitidis* is a Gram-negative pathogen responsible for meningitis and septicemia in humans. Previous genomic analyses predicted the presence of a type II cytochrome c biogenesis pathway but no homologue to DsbE was found. Recently, we have identified the N-terminal domain of the *N. meningitidis* PilB as a DsbE-like periplasmic oxidoreductase. PilB was shown to be a protein implicated in survival of the N. gonorrhoeae (under oxidizing stress conditions) in the presence of reactive oxygen species. It is composed of two other domains that display methionine sulfoxide reductase (Msr) activities. Enzymatic tests *in vitro* showed that the N-terminal domain reduces selectively the Msr activities of PilB. A recent genomic analysis of *N. meningitidis* done by our group revealed the presence of three other DsbEs. This raises the question of the role of these putative DsbEs. Do these DsbEs, provided they are expressed, share a redundant function in *N. meningitidis* or are they involved in different oxido-reduction pathways? Biochemical and functional characterizations of the three putative DsbEs of *N. meningitidis* and of the putative DsbD protein partner have been undertaken. Biochemical, kinetics and functional complementation data will be presented which illus-

trate the identification of the DsbE involved in the cytochrome c maturation process in *N. meningitidis*. The results will be discussed in relation to the known structural specificities of the PilB N-terminal domain.

YSF–89
Structural features of the replication initiator protein RepB of the promiscuous plasmid pMV158

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Rolling circle DNA replication (RCR) is widespread among bacterial plasmids, phages and viruses. Plasmid pMV158 is the prototype of a family of plasmids that replicate by the RCR mechanism. RepB is the replication initiator protein of pMV158 and belongs to the superfamily of proteins with RCR-domains. Upon binding to the DNA, RepB cleaves one strand of the DNA at a specific dinucleotide located in the loop of a cruciform (IR-I) leaving a free 3'-OH end that serves as a primer to start DNA polymerase-mediated DNA synthesis. *In vitro*, RepB interacts with its primary binding site or bind locus and with a region of the nic locus that includes the right arm of IR-I. Binding of RepB to the bind locus seems to facilitate the protein binding to the nic locus, which promotes extrusion of IR-I that contains the substrate DNA to be cleaved. The spatial arrangement of the nick site and the bind locus in the dso, and the ability of RepB to hexamerize are unique features of pMV158 among RCR plasmids. Our proposal is to investigate at a structural level the replication of pMV158 initiated by RepB. The X-ray crystal structure of RepB revealed that the full-length native protein forms a toroidal homohexameric ring. Each RepB protomer comprises an N-terminal domain, with the catalytic activity, and a C-terminal oligomerization domain (OD) which forms a cylinder with a 6-fold symmetry in the hexamer. The electron microscopy 3D reconstruction of RepB bound to its specific DNA strongly suggests that the N-terminal domain contains the DNA-interaction surface and constitutes the origin binding domain (OBD). The purification of the separate OBD and OD domains confirmed that the catalytic activity and the hexamerization potential of the protein could be uncoupled. The OBD is a monomer that retains both the ability to bind to the bind locus and the endonucleolytic activity, whereas the OD is in a hexameric state and lacks the DNA binding and catalytic capacities. Furthermore, substitution of some basic residues of the helix $\alpha 2$ reduces the ability of the OBD to bind the bind locus without affecting its endonucleolytic activity. *In vivo* experiments performed with cross-linking reagents suggest that RepB monomers, dimers and hexamers coexist in pneumococcal cells harbouring pMV158. This result suggests a dynamic assembly of RepB as part of its mechanism of action. A similar process has been proposed for other hexameric RCR and non-RCR viral initiators that are structurally homologous to RepB and share a similar domain organization, although the helicase C-terminal domain is not present in RepB. Finally, we speculate on the existence of several sequential steps leading to the initiation of pMV158 replication and the recruitment of a host helicase that would allow the melting of the DNA and the progression of the replisome complex.

YSF–90
Effect of antioxidants on hepatic cytochrome P450 gene expressions in streptozotocin induced diabetes

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Cytochrome P450s are very large and diverse superfamily of hemoproteins found in all domains of life and diverse array of compounds can influence the relative concentrations and/or activities of P450s. Expression of P450s is modulated not only by exposure to various chemicals, but also by some pathophysiological conditions such as diabetes. Six different Cytochrome P450 isoforms namely 1A1, 2A1, 2E1, 2C11, 3A1, 4A1 were quantified by quantitative Real Time PCR to assess their relative expressions in control and diabetic rat liver tissues. At the same time, effect of lipoic acid (LA), vitamin C (VC) and resveratrol (RSV) on these isoforms were evaluated. Diabetes were induced by streptozotocin (STZ) injection and continued until the end of the 4 weeks. Antioxidants were given 1 week after application of STZ until the end of 4 weeks daily. According to results, diabetes enhanced the CYP1A1, CYP2E1, CYP3A1 expression while decreased CYP2C11, and CYP4A1 and not changed CYP2A1&2 expressions. LA and VC seem to decrease almost all CYP expressions except CYP2C11. Similarly their combined effect was in the same decreasing trend. Furthermore, RSV was seemed to be much more efficient for the lessening of overall CYP expressions but it did not alter the CYP2C11 expression in both control and diabetic animals. Of the six P450 isoforms studied, CYP2C11 familiy is the less effected ones by antioxidiants. To sum up, expressions of cytochrome P450 were altered not only by diabetes but also by exposure to antioxidants such as lipoic acid, vitamin C and resveratrol and expression levels of each isoforms are important factors determining the biological consequences xenobiotics and antioxidant defense systems.

YSF–91
Matrin 3 is an ATM Effector in the DNA damage response

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The DNA damage response is a complex signaling network that is induced by DNA lesions and vigorously activated by double strand breaks (DSBs). The DSB response is mobilized by the nuclear protein kinase ATM, which phosphorylates key players in the damage response pathways. Here, we show that the protein Matrin 3 physically interacts with ATM and is phosphorylated on Ser208 in an ATM-dependent manner in response to DNA damage. Using proteomic analysis we demonstrate that the RNA helicase DHX9, the p53 co-activators hnRNP K, and the DEAD-box helicase DDX5 interact with Matrin 3 in an RNA-dependent manner. Matrin 3's new interactors have established roles in the DNA damage response as well as RNA processing. We show that the loss of DHX9 is associated with increased cellular sensitivity to radiomimetic treatment as measured by clonogenic survival; that Matrin 3 binds the KU70 subunit of the DNA-dependent protein kinase a major player in the nonhomologous end-joining pathway of DSB repair – again in an RNA-dependent manner; and that Matrin 3 associates directly with RNA via the RNA recognition motif 2 (RRM2). Our data also indicate that Matrin 3 is important for proper activation of the DSB-induced cell cycle G2/M checkpoint. The emerging picture is that of a protein complex bound via RNA, whose members

have roles in both the DNA damage pathway and RNA processing.

YSF–92
Role of 4F2hc in tumorigenicity of HeLa cells

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4F2hc (also named CD98hc, SLC3A2, FRP-1), a type II transmembrane protein, is the heavy chain of a cell surface disulfide-linked heterodimer. Originally described as a T-cell activation antigen, it was later shown to be a multifunctional protein involved in cell fusion, integrin signaling and cellular transformation. Moreover, the heterodimers with different light chains in mammals induce amino acid transport. 4F2hc is expressed in all cell types with the exception of platelets, and an increase in its expression has been correlated with development, progression and metastatic potencial of malignant cells. In fact, the expression in tumor cells was correlated with poor patient prognosis; accordingly, 4F2hc was identified as a novel prognostic marker and a potential transmembrane therapeutic target. The aim of this work is to study the role of 4F2hc in cellular transformation and tumorigenesis by means of 4F2hc siRNA repression in HeLa cells. We have analyzed the total and surface expression of 4F2hc in this cell line as well as the glycosylation degree. Furthermore, we have determined the colocalization of 4F2hc with other proteins related to tumorigenicity, as beta1 integrin, by confocal microscopy and co-immunoprecipitation assays. The results suggest that 4F2hc has a role on cellular adhesion, cell transformation and tumorigenesis, probably as a part of a macromolecular unit and via beta1A integrin signalling. To elucidate the role of 4F2hc in tumor development we produced a system to express siRNAs against 4F2hc based on lentiviral vectors derived from HIV (1 positive and 1 scrambled as a negative control). We obtained approximately 80% repression of the target protein in infected HeLa cells. Taking into account that 4F2hc is able to regulate integrin signaling we studied adhesion and spreading of HeLa and siHeLa cells on different components of the extracellular matrix, as laminin, fibronectin, and collagens I and IV; silencing of 4F2hc in HeLa cells alters these processes. In addition, we have evaluated the role of 4F2hc in tumor growth after subcutaneous injection of normal or silenced cells in athymic mice. Silenced cells showed an increase in the latency and a reduction in the size of tumors 24 days post-injection, without significative differences in tumor growth rate.

YSF–93
Differences in glycosylation of serum Prostate-specific antigen subforms could improve prostate cancer diagnosis

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Prostate-specific antigen (PSA) is the tumour marker currently used for prostate cancer diagnosis. However, its low specificity to distinguish between prostate cancer (PCa) and benign prostate hyperplasia (BPH) prompts to the search for better diagnostic approaches. We performed an exhaustive study of serum PSA from 20 PCa patients and 20 BPH patients by two-dimensional electrophoresis (2-DE). Five PSA subforms of different isoelectric point were obtained and the three major ones (F2, F3 and F4) were quantified. Relative percentages of F3 (%F3) and F4 (%F4) were significantly different between PCa and BPH groups. %F3 decreased in cancers and this decrease correlated with the cancer stage, while F4 behaved oppositely. These observations were also found when only focusing on the patients in the low total PSA range 2–20 ng/ml. In addition, %F3 showed a tendency of higher sensitivity and specificity than the currently used total PSA and %free PSA tests. N-glycan analysis of PSA 2-DE subforms (F1–F5) showed that the F3 subform contained higher levels of sialic acid than the F4 subform. Thus, the degree of PSA sialylation may be used to differentiate PCa from BPH. Other changes in the N-glycan profile of PSA, such as a decrease in core fucosylated glycans, were also detected in PCa. Taken together, these changes in PSA associated N-glycans could be used to improve the diagnosis of PCa.

YSF–94
Digestive cysteine cathepsins against celiac disease

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Celiac disease is an autoimmune disorder of the small intestine that occurs in genetically predisposed people. It is caused by an inflammatory response to certain peptides of prolamines from wheat, rye and barley grains, which are resistant to human digestive peptidases. The only effective treatment currently available to a patient is lifelong strict dietary exclusion of prolamine-containing products. The prolamines of wheat grains, gliadins, also serve as the main natural dietary proteins of *Tenebrio molitor*, a stored-product pest. So a specific digestive system of this insect includes peptidases capable of splitting prolamines, and can serve as a source of enzymes hydrolyzing resistant gliadin peptides. Prolamines contain up to 50% of glutamine and up to 30% of proline. The goal of this study was a search for a digestive peptidase in *T. molitor* possessing post-glutamine cleaving activity and characterization of the substrate specificity of this enzyme. Glutamine specific digestive peptidase of *T. molitor* was purified and identified as a cysteine cathepsin L. The purification procedure included gel filtration on Sephadex G-100, anion-exchange chromatography on Mono Q column, and affinity chromatography on soybean trypsin inhibitor agarose. The substrate specificity of the enzyme decreased in the series Z-Phe-Arg-pNA > Z-Arg-Arg-pNA > Glp-Phe-Ala-pNA > Z-Ala-Ala-Gln-pNA > Glp-Val-Ala-pNA > Bzl-Arg-pNA, where Glp-pyroglutamyl, Z-benzyloxycarbonyl, pNA-*p*-nitroanilide. The parameters of lysosomal human cathepsin L substrate specificity were different, and the activity with Z-Ala-Ala-Gln-pNA was 6-fold lower when compared to the maximum activity with Z-Phe-Arg-pNA. The hydrolysis of gliadin by cysteine peptidase was verified using total gliadin preparation and a fluorogenic substrate Abz-LPYPQPQLPQ-EDDnp (where Abz-*o*-aminobenzoyl, EDDnp-ethylenediamine dinitrophenyl), which includes a part of gliadin sequence. It is suggested that digestive cathepsin L of *T molitor* has a potential for oral administration to treat celiac disease.

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YSF–95
The role of distinct CD4+ T cell subsets in protection against tuberculosis infection in mice

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Protection against tuberculosis (TB) largely depends upon generation of IFN-γ-producing $CD4^+$ T cells that activate *M. tuberculosis*-infected macrophages for mycobacterial killing. To improve strategies of TB control it is important to understand differentiation, distribution and regulation of T lymphocyte subsets in lymphoid organs and the lungs of infected host. Recently it was shown that activated population of CD4 T lymphocytes bearing $CD44^{hi}$ $CD62L^{lo}$ phenotype is heterogeneous and consists of $CD27^{hi}$ and $CD27^{lo}$ subsets. Since the relationships between these cells remained unknown, in the present study we performed functional analyses of corresponding lymphocyte subsets in C57BL/6 mice infected with *M. tuberculosis* H37Rv. During infection, highly differentiated $CD4^+$ $CD27^{lo}$ T lymphocytes accumulated predominantly in the lungs (L), whereas their content in the lymph nodes (LN) was low. In agreement with this finding, $CD4^+$ $CD27^{lo}$ T cells readily underwent apoptotic death in the LN, but were relatively resistant to apoptosis in the L. Evaluation of the expression of genes encoding key cytokines (qrt-PCR) demonstrated that the L-located $CD4^+$ $CD27^{lo}$ cells produced significantly more mRNA for IFN-γ and TNF-α compared to their $CD4^+$ $CD27^{hi}$ counterparts. Next, we compared the capacity of the two T cell subsets to activate infected macrophages. Mycobacteria-infected peritoneal macrophages we co-cultured with either $CD27^{hi}$ or $CD27^{lo}$ immune T cells, and after 72-hours we evaluated the degree of inhibition of mycobacterial growth ($[^3H]$-uracile uptake), and the level of NO production by macrophages. Comparison of T-cell/macrophage ratio curves clearly indicated that $CD4^+$ $CD27^{lo}$ T cells were substantially more potent macrophage activators, compared to $CD4^+$ $CD27^{hi}$ cells. Finally, to demonstrate the pivotal role of $CD4^+$ $CD27^{lo}$ cells in protection against TB *in vivo*, we estimated the degree of accumulation of these cells in the L of genetically heterogenous mice. (A/Sn×I/St)F2 hybrids (N = 106) originating from TB-susceptible I/St and TB-resistant A/Sn inbred mice were infected with *M. tuberculosis*, and the degree of the body weight loss by individual mice at week 4 of infection was superposed against the numbers of $CD4^+$ $CD27^{lo}$ cells in their lungs. The degree of wasting reversly and highly significantly correlated with the content of $CD27^{low}$ $CD4^+$ $CD27^{lo}$ cells, suggesting that differentiation of $CD4^+$ cells along the $CD27^{hi} \rightarrow CD27^{low}$ axis is an important factor of protection against TB.

YSF–96
Clusterin as possible biomarker of response to treatment of prostate cancer cells with PolyphenonE

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Background: Green tea consumption has been linked to cancer-preventive action in preclinical studies. Anti-prostate cancer candidate Polyphenon E (PolyE) is a green tea-derived catechin extract (GTE) found safe for humans. We previously found that (1) administration of a similar GTE was effective at inhibiting prostate cancer progression in a selected population of patients bearing High Grade Prostate Intraepithelial Neoplasia (HGPIN); and (2) Clusterin (CLU) expression upregulation and/or nuclear localization of CLU is associated to induction of apoptosis in prostate cancer cells.
Objective: To assess whether GTE administration would affect CLU expression in responsive cells. This finding would provide a possible mechanism for anti-cancer effect of GTE.
Methods: Immortalized human prostate epithelial cells PNT1a and prostate cancer cells PC-3 were used. The IC50 dose of PolyE was determinated and both cell lines were treated for 12–48 hours. CLU protein was detected by Western blot and immunocytochemistry. Apoptosis induction and cell cycle progression were studied by FACS and Western blot. Caspase-3 activity was also measured.
Results: In PNT1a cells PolyE causes cell morphology alteration, inhibits cell proliferation, causes G_0–G_1 cell cycle arrest, inducing cell death by an unknown caspase-3 independent mechanism. Concomitantly a weak increase in CLU cytoplasmic and nuclear staining was observed while PCNA protein expression remained unchanged. PC-3 cells treated with PolyE showed an increase in caspase-3-independent cell death. Cells remained attached to surface showing drastic morphological changes. Nuclear localization of CLU was detected at 24 hours treatment, while specific staining was perinuclear and at the edge of plasmalemma. PCNA protein levels was slightly decreased, consistently with inhibition of cell proliferation.
Conclusions: PolyE inhibits the growth of transformed prostate cells inducing caspase-3-independent cell death. Translocation of CLU to the nucleus was observed under these conditions. Thus, PolyE may act as anti-cancer agent by altering CLU sub-cellular localization and promoting accumulation of pro-death nuclear CLU.

YSF–97
Impact of 5-azacytidine on Oct4 and Nanog DNA methylation/expression in experimental mouse teratocarcinoma

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DNA methylation is an epigenetic mechanism regulating normal embryonal development and development of cancer. Experimental mouse teratocarcinoma connects embryogenesis and cancerogenesis. Gastrulating mouse embryo transplanted at an ectopic site gives rise to teratocarcinoma. Beside differentiated tissue it contains embryonal carcinoma cells (EC). Self-renewal and pluripotency of EC cells seems to be determined by Oct4 and Nanog, members of core transcriptional regulatory circuitry. 5-azacytidine (5azaC), as an epidrug, induces DNA hypomethylation and alters gene expression. This experiment investigates changes in the DNA methylation of Oct4 and Nanog induced by 5azaC in experimental mouse teratocarcinoma, detects consequent modification in gene expression and describes changes in tumor growth. 7.5 days-old C3H embryos were transplanted under the kidney capsule of syngeneic adults. First group of animals was killed after 4 weeks. Other two groups were treated with 5azaC or saline (control) twice a week for next 4 weeks. Cancer samples were isolated and weighted. DNA was isolated. After bisulfite conversion and PCR amplification, DNA methylation of Oct4 and Nanog was analyzed by pyrosequencing. RNA was isolated using commercial kits. Standard protocol for qPCR was performed. Lowest Oct4 and Nanog DNA methylation status was found in

embryos. In 4-week-old tumors DNA methylation was significantly higher and Oct4 and Nanog expression was detected. In 8-week-old untreated tumors (control) methylation was higher compare to 4-week-old tumors. Surprisingly, the expression was significantly higher. In treated 8-week-old tumors methylation was slightly lower than in control. Oct4 and Nanog expression was much lower (more than 50% and < 0.05, respectively) in treated tumors. Highest tumor growth was observed during second 4 weeks and was considerably reduced under 5azaC treatment. Highest DNA methylation level in 4-week-old tumors reflects the differentiation process in teratocarcinoma during its transition from embryo. Expression of stemnes genes corresponds to the high intensity of growth during second 4 weeks which is in concordance to their role in maintaining pluripotency and self-renewal. Epidrug 5azaC significantly reduced Oct4 and Nanog expression leading to growth retardation although without change in their DNA methylation status. This leads to conclusion that Oct4 and Nanog expression may be moderated by a slight DNA demethylation or some other mechanism.

YSF–98
Study of mechanism of Eph receptors activation

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Eph receptors, the largest subfamily of receptor tyrosine kinases, and their ephrin ligands are important mediators of cell–cell communication regulating cell attachment, shape and mobility. Since both Eph receptors and ephrins are membrane-bound proteins, the Eph/ephrin subfamily send information bidirectionally – that is, into both the receptor-expressing and the ligand-expressing cells. The Eph receptor extracellular part (Eph-ECP) contains a highly conserved N-terminal ligand binding domain (LBD), an immediately adjacent cysteine-rich region (CRR) and two fibronectin type III (Fn3) repeats. All ephrins contain a conserved extracellular receptor-binding domain (RBD). The aim of this work is to study conformational changes of murine EphB2-ECP induced by murine ephrinB2-RBD binding.

Objectives: EphB2-ECP (residues 28–550), EphB2-LBD (residues 28–210), EphB2-LBD-CRR (residues 28–330) and their complexes with ephrin-B2-RBD (residues 25–233).

Methods: Circular dichroism measurements. CD spectra were recorded in phosphate buffer (pH 7.0) at protein concentration 0.3–2.0 mg/ml on a J-850 dichrograph. Fluorescence measurements. Measurements were made in phosphate buffer (pH 7.0) on a MPF-44A device. Differential scanning calorimetry. DSC experiments were performed using the DASM-1A microcalorimeter with a cell volume of 1 ml at a heating rate of 0.5, 1.0 and 2 K/minutes. The protein concentrations were between 0.5 and 1.0 mg/ml.

Results: CD spectra has demonstrated an absence of any significant changes in the secondary structure of EphB2-ECP and ephrinB2-RBD due to the complex formation.Fluorescent spectra has shown some changes in the local microenvironment of a Trp residue(s) after formation the complex. DSC has revealed the most interesting data. The temperature of melting (T_m) of the EphB2-LBD as a component of the whole EphB2-ECP is lower (52°C) than for the separated EphB2-LBD (55°C). Hence the EphB2-LBD in the intact EphB2-ECP is destabilized with another domain, most probably with the nearest EphB2-CRR. After binding the ephrin-B2-RBD the stability of the EphB2-LBD as a component of the whole EphB2-ECP is increasing to 55°C. The most surprising result is the significant increasing of T_m (from 66 to 70°C) of the second peak which correspond to the melting of Fn3 domain. This may be explained by dimerization of one of the Fn3 domains.

Conclusions: At least the thermal stability of the EphB2-LBD and one of two Fn3 domains increased. Hence, the Fn3 domains of EphB2-ECP are involved into the receptor dimerization upon ligand binding most probably in zipper-like mechanism.

YSF–99
Study of phospholamban incorporated in nanoBLMs

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Background: Phospholamban (wt-PLN) is an integral membrane protein that regulates cardiac sarco/endoplasmatic CaATPase (SERCA). PLN exists in equilibrium between monomeric and pentameric forms. Monomeric unphosphorylated PLN inhibits SERCA, but when PLN is phosphorylated releases the inhibition and allows calcium translocation. In literature there are four principal proposed structural models of pentameric wt-PLN (1) and it is not clear if the wt-PLN pentamer can form membrane pores.

Methods: We are studying PLN using a new experimental model of biological membrane (nanoBLMs) (2). The properties of the nanoBLMs have been characterized by conductivity and electrochemical measurements.

Results: The conductivity recorded on nanoBLMs containing wt-PLN in the presence of $NaClO_4$ is ~90% of the value in the presence of NaCl. This result is in agreement with the values of equivalent conductance at infinite dilution for Cl^- and ClO_4^-. Moreover it is evident that the conductance in the presence of ChoCl is half of that of NaCl at the same concentration. These results suggest that contrary to Na^+, Cl^- and ClO_4^-, Cho^+ cannot pass through the membrane embedding wt-PLN.

Conclusion: From our experimental data it seems that wt-PLN can form pores which are not selective for small ions. These data are in agreement with the hypothesis of Oxenoid and Chou (3), according to which physiologically relevant ions such as Na^+, K^+ and Cl^- are small enough to pass through the narrowest part of the wt-PLN pentamer (diameter ~ 0.36 nm). This pore dimension is supported by our experimental data indicating that a big organic cation like Cho^+ (radius 0.33 nm) cannot pass through the membrane containing wt-PLN.

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YSF–100
PI-55 is a purine-derived cytokinin antagonist that blocks cytokinin action via receptor inhibition

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Cytokinins, important plant hormones, regulate diverse processes during growth and development. Histidine kinases (HK) have been identified as cytokinin receptors in Arabidopsis and other species. Genetic analyses of cytokinin receptor mutants has

assigned functions to the receptors in the regulation of shoot growth, leaf senescence, seed size and germination, root elongation and branching, and nodule organogenesis. Chemical inhibitors of cytokinin perception would be thus expected to influence plant growth and development and might possess a potential for agricultural application. Synthetic compounds antagonizing action of other phytohormones are commonly used in basic studies of the functions of these hormones and in agriculture. Previously, such compounds have not been available for cytokinin research. We report the identification of the first known molecule antagonizing the activity of the plant hormone cytokinin at the receptor level. This compound, 6-(2-hydroxy-3-methylbenzylamino)purine, designated PI-55, is structurally closely related to highly active cytokinin 6-benzylaminopurine, but substitutions at specific positions of the aromatic side chain strongly diminished its cytokinin activity and conferred antagonistic properties. PI-55 competitively inhibited the binding of the natural ligand transzeatin to the Arabidopsis cytokinin receptors CRE1/AHK4 and AHK3 and repressed induction of the cytokinin response gene ARR5:GUS. Genetic analysis revealed that CRE1/AHK4 is the primary target of PI-55. Cytokinin bioassays also demonstrated the anticytokinin effect of PI-55 in several other species. Furthermore we show that PI-55 accelerated the germination of Arabidopsis seeds and promoted the root growth and formation of lateral roots, thus phenocopying the known consequences of a lowered cytokinin status and demonstrating its potential to inhibit cytokinin perception in planta. Our results indicate that PI-55 acts as an inhibitor of cytokinin receptors and antagonizes cytokinin action *in vivo*. In studies on the functions of cytokinins the compound can be used as a modulator of endogenous cytokinin activity as an alternative to genetic approaches and may have interesting applications as a growth regulator for modifying traits of crop plants. PI-55 is the first example for the targeted development of a cytokinin antagonist and represents an initial step for preparation of cytokinin antagonists with broad activity and reduced agonistic properties.

YSF–101
Application of metadynamics to speed up simulations of conformational changes

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Molecular dynamics simulation is gaining importance in biological sciences.Unfortunately, numerous biomolecular processes, such as proteinfolding, molecular recognition, chemical reactions or conformational changes, are too slow to be efficiently simulated by conventional simulation techniques. Metadynamics method speeds up the simulation by energetically disfavouring states of the studied system that havebeen previously explored. This helps the system to explorehigh-energy states and to cross energy barriers. Here we present an application of metadynamics in the interpretation of gas-phase spectra of a peptide, in modelling of carbohydrate conformations andin conformational studies of a model protein. In the first example it was necessary to prove, that one conformational family of the peptide Gly-Phe-Ala, which has not been experimentally detected, is present under experimental conditions. A recent study indicates, that the fact that this conformation was not detected, can be explained by photochemical reasons. The second example of metadynamics study is amodelling of conformational equilibria in carbohydrates, namely ring puckering and conformations of primary hydroxyl groups of pyranose sugars. These equilibria are discussed in the context of the function of carbohydrate-processing enzymes. Finally, metadynamics has been applied in modelling of conformational dynamics of ubiquitin to compare metadynamics with conventional techniques.

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YSF–102
Involvement of the calpain/calpastatin system in familial amyotrophic lateral sclerosis

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Background: Amyotrophic Lateral Sclerosis (ALS) is an adult-onset disease involving the progressive death of motor neurons in human cortex and spinal cord and as a result, a massive muscle atrophy takes place. A number of fALS (familial ASL) presents mutation in SOD1, leading to structural changes and misfolding defects in the enzyme. This muscle and nerve degeneration has been proposed may be due to a prolonged alteration of Ca^{2+} homeostasis, promoting the activation of the calpain/calpastatin system.

Objectives: Activation of the proteolytic system has been studied in tissues of transgenic mice overexpressing the SOD1-G93A(+) mutant, a model for fALS, in order to identify the mechanism involved in the tissue damages.

Methods: The activation state of the calpain/calpastatin proteolytic system was studied in motor neurons, brain cortex, upper and lower spinal cord and in skeletal muscle. The active calpain forms were identified by confocal microscopy following interaction directly in the tissue with a mAb recognizing the protease in its active conformation. The levels of calpain substrates (NO synthase isozymes and a-spectrin) were also determined. Calpastatin activity, its molecular forms and expression were also characterized.

Results: The extent of the calpain activation was particularly high in the skeletal muscle and in lower motor neurons of spinal cord. An intermediate level of calpain activation was also detected in brain cortex motor neuron. The cerebellum and the other portions of brain resulted less affected. Thus, the observed level of active calpain reflects the alteration in Ca^{2+} homeostasis occurring in the tissues. This conclusion has been confirmed by digestion of calpain substrates (a-spectrin and NO synthase isozymes) and of calpastatin, which is converted to the still active 15 kD species, a process also promoted by active calpain. However, calpastatin activity was found to be decreased at higher extent in those tissues showing also the highest extent of calpain activation. The consumption of this protein inhibitor was partially compensated by an increased calpastatin synthesis.

Conclusion/Application to practice: These observations pointed out that, in this fALS model, the target tissues showed an altered Ca^{2+} homeostasis promoting calpain activation. Since the loss of calpastatin reduces the intracellular inhibitory capacity, the restoration of an efficient calpain regulatory mechanism could improve the survival of neurons and reduce tissue damages.

YSF–103
Expression of dipeptidyl peptidase-IV and related molecules in primary cell cultures derived from human astrocytic tumors

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Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) and related proteases exhibiting similar enzymatic activity, including fibroblast

activation protein-alpha, DPP-8 and 9, hydrolyse N-terminal dipeptides from multiple biologically active peptides like glucagon-like peptides, neuropeptide Y, substance P and some chemokines such as stromal cell derived factor-1alpha (SDF-1). This processing can change signalling pathways triggered by the particular mediator and alter consequent biological responses of the target cells, and is supposed to be important for multiple cellular processes, including cell proliferation, malignant transformation, migration and invasion. In our previous studies, we observed increased DPP-IV-like enzymatic activity in high-grade astrocytic brain tumors compared to non-tumorous tissues. Interestingly, this rise was paralleled by an increased expression of CXCR4 receptor, which together with its ligand SDF-1 is known to be important in gliomagenesis. In this study, using real time RT-PCR, immunocytochemistry, flow cytometry and enzyme activity studies we have analysed expression pattern of DPP-IV, FAP, DPP-8, DPP-9, and CXCR4 in primary cell cultures derived from high-grade astrocytic tumors. Primary cell cultures displayed varying morphology, differed in the expression of DPP-IV and related molecules and DPP-IV-like enzymatic activity. Contrary to the glioma tissue bioptic samples, DPP-IV-like enzymatic activity negatively correlated with CXCR4 mRNA expression. Besides, we observed that higher DPP-IV-like enzymatic activity was associated with slower proliferation of particular cell culture. In addition, negative association of cell growth and DPP-IV-like enzymatic activity was also kept in individual passages along with the culture propagation.

Our results suggest possible role of DPP-IV and related molecules in the regulation of growth of transformed astrocytic cells.

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YSF–104
Hydrogen peroxide inhibits exercise-induced increase of circulating stem cells with endothelial progenitor capacity

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Background: Vascular oxidative stress has been shown to promote many pathophysiologic changes resulting in cardiovascular remodeling underlying cardiovascular diseases. Recent evidences suggest that regeneration of damaged vascular endothelium involves the participation of circulating stem cells mobilized from the bone marrow (EPCs). The number of EPCs was reported to inversely correlate with the number of cardiovascular risk factors and is reduced in cardiovascular disease. Despite recent advances in EPC studies, the molecular mechanisms of EPC mobilization remain unclear.

Objectives: We sought to investigate whether steady state levels of vascular hydrogen peroxide, reactive oxygen species responsible for vascular oxidative stress, impact on exercise-induced EPCs mobilization.

Methods: C57Bl/6 mice, transgenic mice with endothelial-specific overexpression of catalase (cat^{++}) or eNOS ($eNOS^{++}$) and their transgene-negative littermates (cat^{n}, $eNOS^{n}$) and eNOS knockout mice ($eNOS^{-/-}$) were investigated. Mice were randomly assigned to sedentary groups and groups subjected to either freely moving, forced physical activity or voluntary exercise. Selected groups of cat^{++} and cat^{n} were orally treated with the catalase inhibitor aminotriazole. EPCs were measured using anti-mouse CD3, Flk-1 and CD34, CD133 or Sca-1 antibodies.

Results: Three weeks of exercise training failed to increase circulating EPCs defined as double positive for Flk-1 and CD34, CD133 or Sca-1 in freely moving C57BL/6 mice and in mice subjected to forced physical activity or voluntary exercise. Neither insertion of additional genes encoding for catalase (cat^{++}) or eNOS ($eNOS^{++}$) nor eNOS knockout ($eNOS^{-/-}$) changed EPC counts in resting mice. In contrast, inhibition of catalase by treatment with aminotriazole strongly reduced circulatory EPCs in sedentary cat^{n} and cat^{++} ($P < 0.05$, n = 5–8). When cat^{++} mice were subjected to forced or voluntary exercise training, the number of circulating EPCs was strongly increased (n = 4–8, $P < 0.05$), an effect completely inhibitable by aminotriazole.

Conclusion: Our results suggest that hydrogen peroxide, a known component of vascular oxidative stress, likely contributes to the impairment of important stem cell-induced endogenous vascular repair mechanisms in cardiovascular disease. These data suggest a new pathophysiologic role of hydrogen peroxide as one player of vascular oxidative stress, i.e. a reduction of the number of circulating EPCs.

YSF–105
Evaluation of antitumor activity of novel inhibitors of JAK/STAT3 pathway in glioma cells

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Constitutive activation of STATs, in particular STAT3, have been found in a large number of human tumors. Persistent signaling of STAT3 may contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis, thus it emerges as promising molecular target for cancer therapy.We developed a cell-based system for screening the small molecules inhibitors targeting STAT3 signaling and screened 18 compounds synthesized by modifying the structure of WP1066. Compound 2 [(E)-2-cyano-*N*-((S)-1-phenylethyl)-3-(pyridin-2-yl)acrylamide] was found to be the most promising STAT3 inhibitor. Comp2 at doses ≥25 μM significantly reduced the level of phosphorylated STAT3 (Tyr705) as well as phosphorylated upstream regulators JAK1 and JAK2. Treatment with Comp2 at doses ≥25 μM significantly inhibited a viability of rat C6 glioma cells. Appearance of cleaved caspase 3 and PARP, hallmarks of apoptosis, were detected 24 hours after treatment with 25 μM Comp2. Noticeably, we observed a rapid (in 30 minutes) cell detachment from a substratum. To investigate the molecular changes induced by Comp2 downstream of STAT3, the expression of cell cycle and apoptosis regulators was evaluated by real-time PCR. The results show that the expression of genes coding for cyclin D1 and antiapoptotic protein Bcl-XL was significantly diminished in C6 glioma cells treated with 25 μM Comp2. The effect of Comp2 on MAPK and PI3K/Akt signaling pathway were determined. While Comp2 strongly elevated the level of phosphorylated p38, JNK and ERK1/2, phosphorylation of Akt remained unchanged and phosphorylation of focal adhesion kinase (FAK) decreased. The present study demonstrates that selected, newly synthesized compounds inhibit STAT3 phosphorylation and STAT-dependent transcription, affect cell attachment and viability of glioma cells.

YSF–106
Recurrent pregnancy loss: detoxification and blood coagulation proteins involvement

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Recurrent pregnancy loss (RPL) represents an intriguing problem in obstetric practice in which genetic and acquired factors play a role. Risk of RPL associated with exposure to endogenous or exogenous substances. Enzymes such as P4501A1 (CYP1A1) metabolize organic compounds to reactive compounds which damage cells and DNA. *N*-acetyltransferase 2 (NAT2) is involved in the initial biotransformation metabolism of aromatic amines and hydrazines. Glutathione S-transferase (GST) catalyze the binding of a large variety of electrophils to the sulphydryl group of glutathione, and have a main function in binding and transport of a wide variety of harmful compounds. Thrombophilic disorders and hypofibrinolysis are risk factors in women with RPL. During pregnancy, changes in blood coagulation may play a role in the occurrence of abortion, since haemostatic disorders may result in obstruction of placental bed vessels. Factor V Leiden (FVL) gene, is associated with a hypercoagulable state and increased susceptibility for venous thrombosis. Factor II (prothrombin) gene is associated with higher plasma protrombin concentrations, augment thrombin generation and increased risk of venous and arterial thrombotic disease. Aim of this study was to investigate the possible role of and stage detoxification and coagulation systems genes polymorphisms in the pathogenesis of RPL. Above-mentioned proteins encoding genes polymorphism was detected by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The polymorphic variants of those genes were analyzed in 24 women (case group) with RPL and in 171 women (control group) with the uncomplicated obstetric history. The frequency (80%) of NAT2 gene SS genotype in case group was significantly ($P < 0.05$) higher than in control group (57%). The frequencies of GSTM1, GSTT1, CYP1A1, FII and FVL polymorphic variants were practically similar in both analyzed groups. It had been shown that NAT2 S/S genotype really can be involved in the process of RPL, which may be associated with changes in steroid hormones level. From our data the identification of NAT2 S/S genotype can be used as a marker for high risk recurrent pregnancy loss prediction in genetic testing family programs.

YSF–107
Mutp53 non-B DNA structure binding to intronic sequences modulates gene expression in U251 cells

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Mutation of the TP53 gene is a common event in human cancers. Most of all TP53 alterations in human tumors are missense point mutations resulting in the expression of a functionally altered mutant p53 (mutp53) proteins, with an exchange of a single amino acid. Mutp53 proteins have lost sequence-specific DNA binding, but they still have the ability of the structure selective interaction with non-B DNA. In our work, we combined chromatin immunoprecipitation (ChIP) with DNA cloning and sequencing (ChIP-cloning) to identify functional binding sites of mutp53 and provide evidence for the proposed model of mutp53 activity. As proof of principle we established a small library of genomic sequences bound by p53R273H in human U251 glioblastoma cells and confirmed the specific binding of mutp53 to isolated DNA fragments by electrophoretic mobility shift assay (EMSA). ChIP-sequences, like S/MARs, were found to be rich in repetitive DNA sequences and dispersed over non-coding DNA regions. We propose that in U251 cells p53R273H exerts a regulatory effect on gene transcription by acting as DNA/chromatin associated factor that participates in the organization of chromatin into functional domains. Thereby, our approach led to the identification of the PPARGC1A and FRMD5 genes as targets of mutp53 regulated by binding to intronic and intergenic repetitive sequences. Identified repetitive sequences have potential to form non-B DNA structures (e.g. triplex DNA and DNA cruciforms) as we predicted by computational methods and detected by enzymatic methods. With these findings we presume a model that the oncogenic functions of mutp53 can be caused by its interaction with intronic and intergenic non-B DNA sequences and modulation of gene transcription via reorganization of chromatin.

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Reference:

Brazdova M., Quante T., Togel L., Walter K., Loscher Ch., Tichy V., Činčarova L., Deppert W., Tolstonog G. Modulation of gene expression in U251 glioblastoma cells by binding of mutant p53 R273H to intronic and intergenic sequences, *Nucleic Acids Res.* 2008.

YSF–108
Extracellular pH modulates the release of Matrix Metalloproteinase-9 (MMP-9) by stimulated neutrophils and its activity

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Background: Acidosis and alkalosis are complications that may be causes of poor prognosis in critically ill patients. There are evidences that acid–base abnormalities can play a role in immunological disfunctions modifying neutrophils responses that can be affected by changes in the external pH (1). Neutrophils have different kind of granules: primary granules are Myeloperoxidase (MPO) positive and MMP-9 negative; secondary granules with lactoferrin, and a little amount of MMP-9; tertiary granules have a larger amount of MMP-9, that is one of the known effectors of inflammation.

Objectives: The aim of this study is to observe *in vitro* if the MMP-9 released by neutrophils and its enzymatic activity are affected by extracellular alkalosis and acidosis conditions. This may have considerable implications in severity of alkalosis and acidosis.

Methods: Neutrophils were isolated by dextran sedimentation and Ficoll-Hypaque gradient centrifugation. Contaminating RBC were removed by hypotonic lysis. Cell pellets were resuspended in NaCl 0.9% and counted. 2×10^6 cells were placed in RPMI medium at various pH with 1% FBS and LPS 10 mg/ml and placed at 37°C for 30 minutes; conditioned mediums were collected and total MMP-9 was measured with an Activity Assay System.

Results: Extracellular acidosis and alkalosis enhance neutrophil proinflammatory response. We have studied the release of MPO and MMP-9 in the pH range from 7.0 to 7.7. After LPS-stimulation, MPO released doesn't show significative difference in the pH range studied. Instead, the amount of MMP-9 increases both under acidic and basic conditions ($P < 0.001$) compared to physiological pH (7.4). Furthermore, the amount of MMP-9 released at basic pH is greater than that released at acidic pH ($P < 0.001$). We have also analysed the pH dependence of MMP-9 proteolytic activity. We have observed a steady increase of MMP-9 activity from pH 7.0 to 7.7.

Conclusion: Neutrophil response to a proinflammatory stimulus is differentially affected by the environmental pH. The release of primary granules seems to be insensitive in the analysed pH range, while the release of tertiary granules is quite sensitive to small pH variations.The increased release of MMP-9 at higher pHs than physiological together with its proteolytic activity dependence from the pH suggest that MMP-9 could be one of the factors in the adverse prognosis of alkalosis over acidosis conditions.

Reference:

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YSF–109
Assembly, structure and therapeutic use of *in vitro* assembled retroviral particles

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Retroviruses belong to the group of viruses that cause severe diseases such as AIDS or leukemia. Therefore detailed study of their life cycle is critical for designing and developing anti-retroviral drugs. Another challenge is the use of *in vitro* assembled retrovirus-like particles for gene therapy purposes. This system serves as an alternative for utilization of genetically engineered viruses in gene therapies. Its advantage is the lack of particle infectivity and replication. We have focused on several aspects of assembly process of Mason–Pfizer monkey virus (M-PMV) particles. We contributed to the understanding of the role of nucleic acids in this process and using several microscopic techniques also to determination of structural organization of immature particle. We successfully optimized conditions for highly efficient process of *in vitro* formation of spherical particles from purified recombinant structural M-PMV proteins. We found that *in vitro* prepared M-PMV particles can incorporate various polyanions, such as RNA or DNA moreover the presence of nucleic acid highly increases assembly process efficiency. We found that viral structural proteins can be N-terminally prolonged by short oligopeptides and that these peptides are exposed on the particle surface. Thus these particles, that have diameter of about 80 nm, could be used also for therapeutic purposes. Several types of modified M-PMV particles were tested either for immunization or for gene therapy purposes. Monoclonal antibodies against selected oligopeptides were already successfully prepared thus supporting the idea that the presence of viral particles enhances immune response against the antigen presented on their surface. Because the knowledge of structural organization of proteins in the retroviral particle is still not very well described we have focused also on this task. Data obtained using several techniques including transmission electron microscopy, atomic force microscopy and cryo-electron microscopy will be discussed from the perspective of the protein subunit organization and the structure of viral particles.

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YSF–110
CoAA (CoActivator Activator) increases the activity of the ETS-related transcription factor ERM by modulating its sumoylation

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ERM, PEA3 and ER81 belong to the PEA3 group of ETS transcription factors family, which share a common DNA-binding domain(ETS domain) of about 85 residues binding the consensus core sequence 5'-GGAA/T-3'. The PEA3 group members are often over-expressed in different types of cancers that display a disseminating phenotype and over-expression of matrix metalloproteinases (MMP). Experimental regulation of PEA3 group member expression influences the invasive process, suggesting a key role in metastasis. Moreover, in Humans, PEA3 and ERM have been respectively described as poor prognostic factors in ovarian and breast tumors. To regulate their target genes, these transcription factors are subjected to multiple post-translational modifications such as phosphorylation and acetylation. Recently, it has been shown that they are also post-transcriptionally covalently linked to SUMO (Small Ubiquitin-related Modifier). ERM possesses four major functional SUMO-modified sites and their sumoylation negatively regulates ERM transcriptional activity. Moreover, the transcriptional activity is also modulated by ERM interaction with protein partners. So, to better understand the molecular mechanisms involved in the regulation of the PEA3 group member transcriptional activity, it is also important to identify these partners. We developed an experimental approach using biotinylated ERM as an affinity matrix to identify by mass spectrometry potential ERM partners from nuclear extracts. This technique allowed us to identify Co-activator Activator (CoAA) as a new potential ERM partner. CoAA was originally reported as a co-activator of the activator TRBP (Thyroid-hormone receptor binding protein). Importantly, CoAA is over-expressed in a variety of cancers. Inparticular, elevated CoAA transcript expression is present in 60–80% of lung, skin, stomach, and testicular cancer, and CoAA protein over-expression is evident in lung, squamous cell, pancreas, lymphoma, and gastric carcinoma. Moreover, 3–5-fold over-expression of CoAA in NIH-3T3 cells significantly increased their proliferation and promoted colony formation in soft agar assays. We report here that the interaction between ERM and CoAA is confirmed *in vitro* and *in vivo*. We show that CoAA enhances ERM transcriptional activity by modulating the ERM sumoylation level. Similar effects are also observed for PEA3 and ER81 but not for ETS1, another ETS family transcription factor.

YSF–111
Regulation of *Acanthamoeba castellanii* alternative oxidase activity by mutual exclusion of purine nucleotides; inhibitory effect of ATP

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Background: Mitochondria of *Acanthamoeba castellanii* (Protozoa) possess a cyanide-resistant alternative oxidase (AOX) –

energy-dissipating system. The activity of this system, thereby the efficiency of oxidative phosphorylation, is regulated by guanosine and adenosine 5-phosphates in a different way.

Methods: Oxygen consumption and membrane potential of isolated *A. castellanii* mitochondria were measured simultaneously using electrodes sensitive to oxygen and TPP^+ ions.

Results: We found that guanine nucleotides activate AcAOX to a greater degree than adenine nucleotides do, and that nucleotides monophosphates were the most efficient. The extent of the nucleotides influence on AcAOX depended on the assay pH being more pronounced at pH 6.8, which is optimal for the enzyme activity. We demonstrate, for the first time, that ATP has an opposite, inhibitory effect on AcAOX activity. Since the inhibition by ATP was also observed in other protozoan, Dictyostelium discoideum and yeast, Candida maltosa mitochondria, it could be a common regulatory feature of all non-plant AOXs that are purine nucleotide-modulated. Kinetic data show that the binding of GMP (a positive allosteric effector) and ATP (a negative allosteric effector) to AcAOX is mutually exclusive.

Conclusion: Our findings suggest that AcAOX activity in *A. castellanii* mitochondria might be controlled by the relative intracellular concentrations of purine nucleotides, in particular by the concentration of ATP relative to that of guanine nucleotides.

YSF–112
Ecm1 is a new pre-60S ribosomal subunit export factor

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In eukaryotes, ribosomal biogenesis is a highly conserved process that takes place in the nucleus and the cytoplasm. In actively growing cells, like yeast, it was estimated that each NPC (Nuclear Pore Complex) contributes to the export of around 25 pre-ribosomal particles per min. Such an extremely active process depends on several redundant export receptors for the pre-60S particles (Crm1/Nmd3, Mtr2/Mex67, Arx1). We identified a novel pre-60S factor, Ecm1 partially redundant with Arx1 and which becomes essential when the NPC is compromised. Ecm1 depletion, combined with the deletion of any of a number of NPC components leads to pre-60S retention in the nucleus. The functional links between Ecm1, 60S biogenesis, pre-60S export and the NPC were correlated with physical interactions of Ecm1 with pre-60S particles and nucleoporins (Nup100 and Nup192). Our results point Ecm1 as an additional pre-60S nuclear export receptor in Saccharomyces cerevisiae. Multiple pre-ribosomal nuclear export receptors including Arx1 and Ecm1 are thus responsible for efficient pre-60S nucleocytoplasmic transport.

YSF–113
Antioxidant effect of green tea and coenzyme Q10 in human coronary artery endothelial cells

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Background: *Hypoxia*-reoxygenization of the human coronary artery endothelial cells is the most frequently encountered injury in many pathologies of the heart, including myocard infarct. Reoxygenization of the tissue causes the formation of reactive oxygen species (ROS), a source of injury. Epigallocatechin gallate (EGCG) is the most abundant (60%) component of the green tea, showing antioxidant effect through its galloyl group. Polyphenon 60 is the combination of green tea polyphenolic compounds which is minimum 60% of total catechins. Green tea (EGCG and polyphenon 60) and coenzyme Q10 are known by their antioxidant properties.

Objectives: We investigated if these compounds can prevent the damage of human endothelial coronary artery cells (HCAEC) by decreasing ROS in an *in vitro hypoxia-reoxygenization* model.

Methods: HCAEC (Cambrex) were cultured in a seeding density of 10,000 cells/well and four samples were used for each condition tested. Experiments were performed in duplicate. HCAEC were preincubated with EGCG (50, 100, 200 μM), polyphenon 60 (50, 100, 500 μg/ml) or coenzyme Q10 (5,10, 50 μM) for 24 hours and then exposed to 24 hours- (hypoxia + glucose deprivation + incubation) followed by 15 minutes-reoxygenization. After preincubation, we used a hypoxic chamber for forming the hypoxic conditions and used PBS with EGCG, polyphenon 60 or coenzyme Q10 (hypoxia + glucose deprivation + incubation) and then let the cells reoxygenate with Endothelial Growth Media (EGM-2) in a CO_2 incubator. We then measured the reactive oxygen species (ROS) fluorometrically: a non-fluorescent hydroxyphenyl fluorescein (HPF) probe become fluorescent in the presence of ROS. Fluorescence excitation and emission maxima are 490 and 515 nm, respectively.

Results: When optimising the time that caused ROS to increase, we showed that whenever reoxygenization period was extended from 1 to 4 hours, ROS increased ($P = 0.029$) and from 4 to 18 hours, again, ROS increased ($P = 0.029$). 24 hours hypoxia-15 minutes reoxygenization and 24 hours hypoxia-3 hours reoxygenization caused ROS to increase compared with the control groups ($P = 0.029$, $P = 0.029$) but 24 hours hypoxia-1 hour reoxygenization didn't change the ROS levels ($P > 0.05$). The concentrations of EGCG and polyphenon 60 tested didn't cause ROS to decrease ($P > 0.05$). On the other hand, coenzyme Q10, at dose of 5 μM, decreased the ROS according to the 24 hour hypoxia-15 minutes reoxygenization condition ($P = 0.029$).

Conclusion: 5 μM coenzyme Q10 is effective in decreasing the ROS production of HCAEC in the *in vitro hypoxia-reoxygenization* model used.

YSF–114
Comprehensive conformational analysis of canonical and modified nucleosides as a key to mechanisms of their biological influence: ab initio study

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The aim of this work was to present a comprehensive investigation of structural and vibrational properties of some nucleosides (canonical, minor and modified) by ab initio calculations. The obtained results demonstrate a wide scope of conformational flexibility of nucleosides corresponding to a narrow range of energy (7–10 kcal/mol). Slightly less than one hundred conformers of deoxyribonucleosides are stabilized by some hundred intramolecular H-bonds of various types, i.e. from the strongest (OH...O), to the weakest ones (involving CH groups). It was shown that at 298.15 K, all of the studied nucleosides are characterized by quasi-degenerate global minima on the Gibbs energy

landscape, each minimum corresponds indeed to two conformers. Conformational equilibrium of isolated nucleosides appears to be shifted towards syn base orientation. Moreover, South (S) conformers of sugar dominate over North (N) conformers. The whole set of conformational parameters were estimated and correlations between them were established. Geometrical, vibrational, structural-topological, and energetic features of intramolecular H-bonds in the calculated conformers were determined. Convolution of calculated IR spectra in the ν(O–H) range for all the thymidine and 2′-deoxyuridine conformers appeared to be consistent with the observed low-temperature matrix spectra. Among the full calculated conformational families of nucleosides, only three DNA like conformers were detected related to BI, A and BII forms. In contrast, 2′-deoxy-6-azacytidine (i.e. modified analogue of 2′-deoxycytidine) adopts only an A form like conformation. A close inspection of the above-mentioned theoretical data leads to some conclusions at biological level. In particular, the biological effect of this modified nucleoside may correspond to the inhibition of replicative DNA polymerase caused by an unusual orientation of the sugar residue against the base in the only A form conformer. On the other hand, the close similarity between calculated energetic and geometric characteristics of thymidine and 2′-deoxyuridine DNA like conformers with anti and syn base orientations and transition states of anti → syn interconversion between them, leads to think that the uracil mismatch glycosylase discriminates between the two nucleosides due to the difference in their shapes (methyl group in thymidine instead of hydrogen atom in 2′-deoxyuridine at position 5 of pyrimidine ring), rather than due to the electronic influence of methyl group

YSF–115
Monitoring interactions between cytokinins and cytokinin binding proteins by fluorescence correlation spectroscopy

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Cytokinins are essential plant hormones involved in cell division, shoot initiation, leaf and root differentiation, photomorphogenesis and senescence. Cytokinin binding proteins (CBPs) have been identified in many species, from mosses to higher plants. Unfortunately, the biological function of these proteins is still unclear, with the exception of cytokinin receptor – CRE1, and some proteins of PR-10 class. Identification and characterization of new CBPs is important to better understand the mechanism of cytokinin action. Fluorescence correlation spectroscopy (FCS) allows the investigation of intermolecular interaction in solution. During FCS measurement, fluorophores are excited and the fluorescence intensity can be measured. The autocorrelation function of the time-dependent fluorescence intensity provides information about fast- and slow-diffusing populations which is translated into the relative amount of bound and unbound species. For our studies we first designed and synthesized two fluorescent cytokinin derivatives. We attached two fluorophores, NBD (7-chloro-4-nitro-2,1,3-benzoxadiazole) and Rhodamine B, to one of the most active urea-type cytokinin 4PU (*N*-phenyl-*N*′-(4-pyridyl)urea). The binding behavior of fluorescent cytokinins to the proteins was investigated by FCS experiments with several proteins. First, we found that the fluorescent probe did not bind to any accidental proteins like BSA, lysozyme or proteinase K. Second, we asked if fluorescent cytokinin would bind to cytokinin-specific binding protein from *Vigna radiata* (VrCSBP). Indeed, this proteins bound fluorescent probe strongly whereas rhodamine B moiety did not interact with VrCSBP at all. A diffusion coefficient range from 2.24×10^{-6} cm^2 s^{-1} (free state) to 0.75×10^{-6} cm^2 s^{-1} (bound state). The protein binding was saturated at a concentration of about 1.7 μM, and a Kd value of 628 ± 8 nM was obtained by scatchard analysis of the binding data. Using a competition assay we determined a dissociation constants of a non-fluorescent cytokinins. Inhibition curves were obtained for natural cytokinin zeatin and for two synthetic cytokinins: 4PU and even more active 4PU derivative – 4PU30 (*N*-(2-chloro-4-pyridyl)-*N*′-phenylurea). Summarising, we demonstrate here fast and precise tool to investigate interaction in solution between cytokinins and any soluble protein of interest.

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YSF–116
Functional studies of EDC3 provide new insights for its role in mRNA degradation

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In eukaryotic cells, gene expression can be modulated at several post-transcriptional steps including mRNA translation and degradation. In the budding yeast Saccharomyces cerevisiae, translation and degradation are intertwined and compete with each other. Intracellular structures called P-bodies, have been implicated in mRNA storage, mRNA degradation, and translational repression. These mRNP aggregates contain translationally repressed mRNAs and proteins involved in translation repression and mRNA degradation. One of the conserved components of the P-bodies, Edc3, was shown to be involved in specific mRNA decapping mechanisms. We developed a sensitive and efficient method that allows measurements of interactions of genetic networks and functional modules at a genomic scale in yeast. A double mutant library is obtained by mating and sporulation. The abundance of individual double mutants in the query population relative to a reference mutant population is analyzed on glass slide microarrays that detect and quantify the tags marking each deletion of the library. To better understand the biological function of Edc3, we identified its functional partners based on the results of a genome wide genetic screen. One of the gene deletions that was synthetic slow growth with the edc3 delta mutation was scd6 delta. By performing functional analysis, we could show that Edc3 and Scd6 are paralogs and play redundant functions in 5 prime to 3 prime mRNA degradation. These results show that, while Edc3 is not an essential protein, it shares with Scd6 an important function for 5 prime to 3 prime mRNA degradation.

YSF–117
Deficiency of respiratory chain complex I in oncocytic tumors

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Many solid tumors exhibit a shift in energy metabolism from aerobic oxidation in the mitochondria to anaerobic glycolysis. Disruptive mutations of the mitochondrial genome (mtDNA) have

been reported for mitochondria rich (oncocytic) tumors of the thyroid and kidney. Recently, we have demonstrated that mutations of the mtDNA in the renal oncocytoma cause a lack of assembled complex I, resulting in undetectable or severly reduced enzymatic activity of complex I. To elucidate if complex I deficiency is a general feature of oncocytic tumors, we performed sequence analysis of the mtDNA and quantified complex I, complex III and complex V in oncocytomas of the parathyroid (n = 6), parotid gland (n = 4), pituitary gland (n = 2), the eyelid (n = 1), adrenal gland (n = 1) and salivary gland (n = 1) by immunohistochemical staining. We observed a lack of complex I in 14/15 oncocytomas, while complex III and complex V were considerably up-regulated. Pathogenic mutations have been found in mitochondrially encoded subunits of complex I. In summary, oncocytomas, independent of their localization, seem to be characterized by a loss of complex I and a compensatory hyperproliferation of mitochondria.

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YSF–118

*S*tructures of coagulation factor IXa mutants with enhanced catalytic activity: implications for the Xase complex

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Background: Coagulation factor IX plays a major role in the regulation of the blood clotting cascade as this protein is situated at the convergence-point of the extrinsic and the intrinsic coagulation pathway. This critical position is emphasized by several mutations in factor IX which are well known to be responsible for X-linked recessive Hemophilia B, a hereditary bleeding disorder. The activity of this extra-cellular, disulfide-carrying proteinase needs to be strongly regulated at several levels e.g. through proteolysis and cofactor-dependence. Only binding of the proteolytically cleaved enzyme to the activated cofactor factor VIIIa and presence of the biological substrate factor X will result in physiologically relevant Xase complex formation.

Objectives: Although factor IX has been intensively studied during the last decades and is in clinical use in plasma-purified as well as recombinant form, the structural basis of factor VIIIa binding and the associated conformational changes leading to the dramatically increased substrate turnover in the Xase complex are poorly understood. Earlier studies (1) identified several factor IX mutants with enhanced catalytic activity when compared to wild-type protein which may serve as a model for these physiological rearrangements. Here we present our recent results in production, purification, crystallization and structure determination of these mutants.

Methods: We established recombinant factor IX expression in *E. Coli* leading to inclusion body formation followed by an optimized, oxidative *in vitro* protein folding approach with simultaneous formation of seven disulfide bonds. Biochemical characterization of the purified proteins indicates correct folding through enzymatic activity demonstrating the suitability of this folding-technique for disulfide-linked coagulation factors. Applying robot-assisted crystallization screening, we were further able to crystalize these highly active mutants and to determine the 3D-structure through a molecular replacement approach.

Results/Conclusion: Based on the 1.5 and 1.9 structures we can now rationalize on the molecular background of activity enhancement and pinpoint putative regulative motifs in coagulation factor IXa. These structures enable us to model the mechanism of activity enhancement in the Xase complex.

References:

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YSF–119

Apoptosis of leukemia cells triggered by purine derivatives combined with mafosfamide – advantage of R-roscovitine

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In present study, we investigated molecular mechanism underlying apoptosis elicited by cladribine, fludarabine, mafosfamide (active form of cyclophosphamide, M) used solely, and combinations, i.e. CM, FM with emphasis on expression of protein involved in apoptosis (Bax, Bak, Mcl-1, cytochrome c, caspase-3 and -9). Additionally, B-CLL cells were exposed to a new cell cycle inhibitor – R-roscovitine (purine-derivative; R) alone as well as in combination with mafosfamide (RM). We have shown that R-roscovitine reduces mononuclear cell viability more efficient than the other tested agent solely, even combinations: CM and FM (Vybrant apoptosis assay #four). Moreover, both R-roscovitine and other examined agent(s) disturb the balance between pro- and antiapoptotic Bcl-2 family proteins in leukemic cells and trigger their apoptosis by a mitochondrial pathway that was confirmed by cytochrome *c* translocation as well as procaspase-9 and -3 proteolysis. Apoptosis of leukemic cells induced by studied agents used individually and in combinations was accompanied by DNA fragmentation (apoptotic ladder), verified by measuring the increase in sub-G1 DNA content compared to untreated cells. We have also observed that used agent(s) affected expression and functional status of cell cycle-related proteins (cyclin E, CDK2, $p27^{KIP1}$). Our data indicate that R-roscovitine seems to be a most potent chemotherapeutic agent due to its dual action affecting both leukemia cell populations: dividing cells by inhibition of cell cycle modulators and quiescent cells by induction of apoptosis.

YSF–120

Production of biologically active human granulocyte and granulocyte macrophage colony-stimulating factors in *Nicotiana benthamiana*

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The human granulocyte and granulocyte macrophage colony-stimulating factors (hG-CSF and hGM-CSF) are glycoproteins with important clinical applications for the treatment of neutropenia, aplastic anemia and reducing infections associated with bone marrow transplants. We evaluated the potential for using a crucifer tobacco mosaic virus (crTMV) viral vector system for efficient expression of the biologically functional hG-CSF and hGM-CSF proteins in Nicotiana benthamiana leaves. The hG-CSF and hGM-CSF genes were cloned into crTMV viral expression vector, driven by A.thaliana Actin promoter. Gene transfer was accomplished by agroinoculation. Five days after inoculation, the maximum concentration of target proteins in leaves was detected. Protein purification was carried out by Ni-NTA affinity

chromatography. The yield of purified hG-CSF was 100 mg/kg of fresh leaf weight. For hGM-CSF – 50 mg/kg. Plant-made hG-CSF stimulated the proliferation of murine bone marrow cells in the same rate as commercial drug (F. Hoffman-La Roche). Plant-made hGM-CSF stimulated the proliferation of human TF-one cells and was as active as commercial drug (Immunex). These results indicate that our methods of expression and purification do not affect the physiological activity of the cytokines. This technology of fast and inexpensive production of hG-CSF and hGM-CSF could be a rational alternative for the existing expensive methods of its production.

YSF–121
A novel pipeline for automated assembly and analysis of high throughput cDNA libraries

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In recent years increased interest in identification of novel functional transcripts can be observed. Fast development of high throughput DNA sequencing methods allowed the scientist for more complex investigation of cell's transcriptome dynamics in response to various stimuli, like stress or infections. However analysis of huge amounts of data produced by such approaches requires highly specialized tools in order to fish out the interesting cases from hundreds of thousands of sequences of transcriptomic 'noise'. Presented pipeline consist of number of well known as well as newly designed tools. First step stands for highly efficient cleaning of raw sequences and their assembly into representative contigs. It is followed by identification of known transcripts, like mRNAs, tRNAs etc. The remaining intergenic sequences are then submitted in the next step to function prediction modules, including prediction of antisense interactions, novel snoRNAs, microRNAs and others. The main advantage of the pipeline is possibility of using it in a fully automated way. In this mode, a set of optimized parameters for every step of the analysis is used. Thus, involvement of the user can be restricted to providing the raw sequencing data. High efficiency of this approach has been achieved due to rigorous cleaning steps, resulting in high quality of sequences used for assembly and then, function prediction. Presentation of the results allows easy and convenient verification of the predictions and identification of sequences of interest.

YSF–122
Complex studies of interactions between flaviviruses and laminin binding protein

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Background: Flaviviruses (West Nile virus and tick – borne encephalitis virus) (WNV, TBEV) are human neuropathogens caused severe meningoencephalitis with high mortality rate. Flaviviral surface glycoprotein E (gpE) mediates viral attachment to host cell co/receptors. Immunochemical data attest an effective and highly specific interaction of the TBEV and Dengue viruses gpE domain II (DII) with the laminin binding protein (LBP). Based on a highly conservative structure of the DII in different flaviviruses we propose a similarly effective interaction between the LBP and the WNV gpE DII.

Objectives: Research for flaviviral gpE interactions with LBP by complex of immunochemical and biophysical methods.

Methods: ELISA and Western blotting: recombinant LBP was tested for attachment to WN virions that were sorbed into polysterol plates in ELISA (100 ng/per well) as well as immobilized into nitrocellulose membrane (500 ng/per lane). Single molecule force spectroscopy (SMFS): measurements were done accordingly established technique (Chtcheglova & Dietler, 2003). AFM Nanoscope IV Picoforce (USA) and AFM SolverP47Bio (Russia) were used. Recombinant polypeptides mimicking flaviviral gpE domains were attached onto tip and immersed into a solution of LBP (250 mg/l) for 15 minutes. The value of the signal jump at the moment of this rupture is taken as a value of the specific interaction force.

Results: The specific binding between WNV gpE and LBP was confirmed by immunochemical and SMFS methods. The recombinant polypeptides modeling WNV gpE domen II (DII) interacted with LBP in ELISA and immunoblotting. The average values of specific interaction force equal to 105 ± 20 pN (single interaction) and 210 ± 50 pN (double interaction) were determined for the LBP and WNV gpE DII pair. Similar force spectroscopy data were obtained for the LBP and TBEV gpE DII interaction.

Conclusion/Application to practice: X-ray model of gpE let us suppose that bc-loop (73–89 aa) of domain II interacts with LBP and together with cd-loop (fusion peptide) determines an initial stages of flaviviral penetration into cell. Mapped anti – receptors gpE epitopes can be assumed as new targets for the blocking of the flaviviral pathogenesis

YSF–123
Prevalence of intrinsic disorder in the Hepatitis C Virus ARFP/Core+1/S

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The Hepatitis C Virus (HCV) Core + 1/S polypeptide, also named ARFP/S is the major form expressed from the alternative reading frame within the Core coding region of Hepatitis C virus genome. Core + 1/S is expressed due to internal initiation at AUG codons (85–87) located downstream of the polyprotein initiator codon. Recombinant Core + 1/S protein was expressed and purified from *E. coli* in native conditions and was shown to react with sera of HCV-positive patients. We subsequently investigated the biochemical and structural characterization of Core + 1/S protein. The conformation and oligomeric status of Core + 1/S protein was investigated using size exclusion chromatography, dynamic light scattering, nuclear magnetic resonance, fluorescence and circular dichroism spectroscopies. We showed that monomers and multimers of Core + 1/S coexist in solution in equilibrium. Consistent with disorder predictions, Core + 1/S monomers lack significant tertiary structure *in vitro* and are mainly disordered under native conditions, whereas evidence of secondary structure elements were found in the Core + 1/S multimers. In the context of the mild detergent OG, we observed an increase of α-helical content. These properties might be functionally relevant for the recognition of diverse molecular partners and/or for the assembly of Core + 1/S. This study is the first reported structural and functional characterizations of the HCV Core + 1/S protein.

YSF–124
Effects of dipeptidyl peptidase-IV overexpression on glioma cells

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Dipeptidyl peptidase-IV (DPP-IV, CD26, EC 3.4.14.5) is a serine protease that regulates a number of mitogenic peptides involved in cancer development such as neuropeptide Y, stromal cell-derived factor-1alpha, substance P (SP) etc. Our previous results demonstrated that DPP-IV is expressed in glioma cell lines *in vitro* as well as in human gliomas *in vivo*. The aim of this study was to (i) investigate the effects of DPP-IV on growth properties of glioma cells using transfectants with mifepristone-inducible DPP-IV expression, and (ii) elucidate the possible underlying mechanism, in particular modification of the SP pro-oncogenic signaling demonstrated previously in malignant gliomas. SP had, via its cognate receptor NK1, mild growth promoting effect in U373 glioma cells as evidenced by an increase of cells in S phase of the cell cycle. Using the ratiometric indicator Fura-2, we observed that SP triggered calcium signaling in U373 cells. The rise of intracellular calcium was lower in U373 cells overexpressing DPP-IV, but this could not be reversed with a DPP-IV inhibitor. Overexpression of DPP-IV in glioma cells led to substantially decreased growth that could also be observed in another glioma cell line T98G with very low intrinsic expression of the SP receptor. Flow cytometric analysis revealed a decrease of cells in S phase of the cell cycle and a G2/M cell cycle block, which were not influenced when cells were grown in the presence of a DPP-IV inhibitor Diprotin A. Cells highly expressing DPP-IV also exhibited decreased migration and adhesion. In conclusion, using transfected glioma cells with mifepristone-inducible DPP-IV expression, we demonstrate that DPP-IV impairs the growth of glioma cells and may alter intracellular signaling cascades triggered by SP in U373 cells. Moreover, our data suggest that the anti-oncogenic effect of DPP-IV in glioma cells may be independent of its enzymatic activity.

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