Session 15. Biotechnology

Lectures

L15.1

Restriction enzymes for RNA?

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

Based on structural analysis of enzymes from the RNase III superfamily we identified loops in the protein sequence that could be extended to make specific contacts with bases in the dsRNA substrate. Biochemical characterization of selected members that possess extended versions of such loops revealed that some of them indeed exhibit sequence specificity. The obtained prototype RRNases recognize a partially degenerated hexanucleotide target sequence and are capable of cleaving individual sites in long dsRNA molecules. For one of such enzymes we solved the crystal structure and constructed a structural model of a protein-RNA complex.

A prototype RRNase that cleaves the RNA strand in DNA-RNA hybrids 5 nucleotides from a nonanucleotide recognition sequence was constructed by fusing two functionally distinct domains: a non-specific RNase HI and a zinc finger that recognizes a sequence in DNA-RNA hybrids. The optimization of the fusion enzyme's specificity was guided by a structural model of the protein-substrate complex and involved a number of steps, including site-directed mutagenesis of the RNase moiety and optimization of the interdomain linker length.

Potentially, RRNases may be used *in vitro* for production of RNA molecules with defined length and termini, which may be a cheaper alternative to chemical synthesis; they may be also used *in vivo* for targeted RNA degradation.

L15.2

Non-enzymatic method of affinity tag removal based on nickel-dependent peptide bond hydrolysis

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The nickel-dependent peptide bond hydrolysis was discovered serendipitously during our studies on molecular mechanisms of nickel toxicity. In follow-up studies on model peptides and peptide libraries we characterized a family of hydrolytically active sequences. Their overall pattern is Raa-Ser/Thr-Xaa-His-Zaa (Raa,Xaa,Zaa - any amino acid, except of X = Pro or Cys), and the hydrolytic cleavage occurs invariably between residues Raa and Ser/Thr. Large differences of reaction rates were found for various Xaa and Zaa substitutions, with a preference for bulky or aromatic residue in these positions.

We also elucidated the molecular mechanism of this reaction. Its first step is the formation of a square-planar complex containing the Ni(II) ion bonded to the imidazole nitrogen of the His residue and three preceding amide nitrogens. Then the reaction proceeds by the N-O acyl-shift resulting in formation of the intermediate oxygen ester with the Ser/Thr hydroxyl, which decays spontaneously in the presence of water, to yield two reaction products: the N-terminal sequence terminated with Raa, and the C-terminal sequence containing the Ni(II) ion bound to the Ser/Thr-Xaa-His moiety.

These studies indicated that Ni(II) hydrolysis reaction has a sufficient sequence specificity to be applied in biotechnology. We developed it further for affinity tag removal in protein purification procedures. For this purpose we obtained several recombinant fusion proteins, equipped with a C-terminal His-tag, preceded by a Ni(II) susceptible Ser-Arg-His-Trp sequence. The proteins were purified with yields of 80–90% at pH 8.2, in a practically useful time scale, hours to days, depending on the temperature of incubation (40–50°C) (Kopera *et al.*, 2012, *PLoS ONE* **7**: e36350).

We demonstrated that the purification can be performed in the presence of reducing agents, denaturants and non-ionic detergents. These results indicate that our method provides a sound alternative for current protein purification procedures. Control experiments with several natively folded proteins, such as ubiquitin, albumin and amylase showed that hydrolysis does not occur if the susceptible sequence belongs to a secondary structural motif. Therefore, the C-terminally engineered sequence will be the sole cleavage site in most recombinant proteins.

Currently this low-cost, non-enzymatic method is directly applicable to thermostable proteins. We work on lowering the temperature of the process in order to expand the range of its applications.

L15.3

Adult stem cells: hopes and hypes of regenerative medicine

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Stem cell is unspecialised cell which has the capacity of self-renewal and, when appropriately stimulated, differentiates into the specific cell type(s). Accordingly, pluripotent stem cells, like embryonic stem cells or induceed pluripotent stem cells give rise to cells of all three embryonic lineages. Multipotent stem cells, such as hematopietic cells of the bone marrow differentiate into all blood cells. Numerous types of unipotent cells (progenitors) form the mature cells of one type, like eg. satellite cells of the skeletal muscle. Adult stem cells located in organs are multipotent (like hematopietic or mesenchymal stem cells of the bone marrow) or unipotent.

There are several examples of succesful application of stem cells in regenerative medicine. More than milion of patients worldwide have already benefited from bone marrow transplantations performed for treatment of leukemias, anemias or immunodefciencies. Skin stem cells are used to heal severe burns, while limbal stem cells of the eye can regenerate the damaged cornea. The big potential and large hopes are linked to the application of pluripotent stem cells, however, their succesful usage will require overcoming numerous practical drawbacks. Due to the latter, also connected to ethical issues associated with embryonic stem cells, the „off label" applications of adult stem cells are undertaken. Those attempts are often linked to claims on the presumed „pluripotent" characters of some of adult stem cells. However, despite strong hopes, tightly bound to generated hype, the properties of such events like „very small embryonic stem cells" or „multipotent adult progenitor cells" have not been confirmed in stringently performed independent studies. Also the notions on the plasticity of bone marrow cells, suggested to differentiate into eg. cardiomyocytes, appears to be not justified, and their therapeutic effect, if any, is rather linked to the paracine properties, i.e. the secretion of various mediators by injected cells, not their differentiation into specialized cell types.

In this lecture the potential of stem cells will be discussed and the results, including own, will be presented critically in light of current understanding of the stem cells' properties.

L15.4

Lactic acid bacteria as a delivery vehicles for vaccine development

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The lactic acid bacteria (LAB) constitute a very heterogeneous group of Gram-positive, non-sporulating, low-GC-content microorganisms. Over the past decade, there has been increasing interest in the use of lactic acid bacteria as mucosal delivery vehicles. These bacteria represent an attractive alternative to vaccinations that employ attenuated bacterial pathogens because they eliminate the potential risk of reversion of an attenuated pathogen to the pathogenic wild type phenotype. There are two strategies used to present heterologous antigens on the surface of LAB cells. The first approach makes use of the C-terminus of a cell–anchoring proteins, which contain LPXTG motif. This mechanism requires processing by a sortase for the covalent anchoring of the protein to the cell wall peptidoglycan. The second approach bases on the peptidoglycan-binding domain (PA-protein anchor) of the major *L. lactis* autolysin, AcmA. AcmA contains three domains: the N-terminal signal sequence, an active site domain and a C-terminal peptidoglycan-binding domain (PA). The PA comprises three LysM motifs. After secretion, AcmA is directed to the cell wall and its C-terminus determines the non-covalent binding to cell wall peptidoglycan. Apart from live LAB cells also nonliving, TCA pretreated, cells (GEM particles - grampositive enhancer matrix) deprived of some surface components and intracellular content constitute safe and efficient vaccine delivery vectors for heterologous proteins. The overall goal of the research conducted in our lab is construction of an effective and safe anti-*Campylobacter* vaccine. Campylobacter spp. are generally regarded as the most common bacterial cause of gastroenteritis worldwide. Campylobacteriosis usually occurs sporadically and results from consumption of contaminated food, mainly poultry meat. Existing approaches to reducing on-farm *Campylobacter* carriage in chickens have had limited success. Consequently, innovation in this area is still needed, especially because, as yet, no chicken anti-*Campylobacter* vaccine is commercially available. An attempt to generate LAB surface display system for *C. jejuni* antigens will be presented.

Oral presentations

O15.1

Potential modulation of biological effects of anticancer drugs and other xenobiotics by their entropically-driven aggregation with fullerene C_{60}

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Fullerenes, carbon nanostructures extensively studied for past 20 years, are believed to possess numerous qualities that may be explored especially in medicine to improve cancer prevention and therapies. However, despite promising observations of fullerene C_{60} activity (including malignant tumors growth suppression), little is known about mechanisms that may lead to noted effects. It is suggested that aromatic rings present in chemical structure of fullerenes may be involved in formation of stacking aggregates with aromatic ligands. This property may be exploited in cancer treatment that would employ fullerene molecule as drug carried that may be adsorbed on cell surface. Additionally, the same mechanism can be used in chemoprevention against aromatic mutagens present in diet. Such effects were described for numerous aromatic xenobiotics and methylxanthines (MTX) with two main mechanisms discussed – interceptor model in which MTX particles sequestrate xenobiotics in mixed stacking aggregates and protector model in which MTX molecules bind to DNA reducing number of binding sites for xenobiotics. In case of fullerenes, due to particle shape and size, only the first proposed mechanism may apply. However, due to presence of tightly packed hydratation shells around fullerene C_{60} molecules, the reaction is suspected to be driven entropically with enthalpy changes oscillating around zero. This work presents analysis of fullerene C_{60} interactions with chosen xenobiotics and their possible biological effects. UV/Vis spectroscopy studies and calculations based on diverse statistical-thermodynamical models were accomplished to show interactions in the mixtures of xenobiotics and fullerene C_{60}. These interactions were assessed as well with employment of ITC. Additionally, in order to investigate biological effects of observed interactions, mutagenic activity of chosen xenobiotics in the presence of fullerene C_{60} was analyzed *in vivo* in the Ames test.

O15.2

Bioengineered spider silk proteins form microspheres for delivery of chemotherapeutics

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A drug carrier should meet several requirements such as: 1) biocompatibility and biodegradability, 2) easy and efficient synthesis procedure 3) nano- or sub-microscale size; as well as 4) ability to bind and release a therapeutic agent. The biomaterial used to form a drug delivery vehicle should also be durable enough to transport an active compound within an organism. Spider silk proteins possess the properties of an ideal drug delivery vehicle: durability, biocompatibility, ability to self-assemble into sub-micrometric spherical particles [1]. Moreover, they can be modified, at the DNA-level by adding a new functional domain responsible for binding a drug or a cell receptor.

The objective of the study was to produce the bioengineered spider silk biomaterial by expression in bacterial culture with efficient purification technique [2], to form the drug carrier – spheres and to study the loading and release-behavior of the chemotherapeutic agents.

The bioengineered spider silk proteins: MS1 and MS2 were derived from the two major spidroins of *N. clavipes* spider: MaSp1 and MaSp2, respectively. Recombinant proteins were produced in *Escheirchia. coli* and purified by a thermal extraction method [3]. The spherical particles were produced by mixing of a soluble silk protein and potassium phosphate at different conditions. The physical properties of the silk spheres were characterized using SEM, FTIR and zeta potential measurements. Cytotoxicity of the spheres was measured by a MTT assay. Drugs: doxorubicin, mitoxantrone and etoposide were loaded into the particles using two loading methods named after-loading and pre-loading. Drug loading and release were studied spectrophotometrically.

The MS1 and MS2 proteins formed microspheres of different morphology and size depending on the processing conditions. The size of the particles was controlled by the concentration of the silk protein, while the phosphate concentration influenced stability of the silk spheres. The MS1-spheres presented a positive, while MS2 spheres a negative zeta potential. Both particle types did not show cytotoxicity towards 3T3 fibroblasts *in vitro*. MS1 particles showed stronger affinity to the two of the studied chemotherapeutics: doxorubicin and mitoxantrone. Doxorubicin and mitoxantrone showed different loading efficiency depending on the applied loading method. Doxorubicin showed a pH-dependent release profile.

The bioengineered spider silk proteins form stable, non-toxic spherical particles which can incorporate an anti-cancer drugs and release it in a pH-dependent manner. By adding the domain responsible for targeting the drug-loaded particles to the selected cell type, the bioengineered silk spheres would provide an effective drug delivery system.

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O15.3

Adhesive and muco-adhesive properties of *Lactococcus lactis* as important factors for its potential health-promoting applications

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In the gastrointestinal tract (GIT), adhesion is a prerequisite for bacterial colonisation. Although lactococci are not a frequent natural element of the intestinal microbiota, they can be used in food (probiotics) and health-related applications (mucosal vaccine, therapeutic drug delivery), both involving their adhesive properties. Bacteria can adhere to the different components of the intestinal mucosa, in particular mucins and proteins of the extracellular matrix (ECM), such as laminin, collagen and fibronectin. Various cell surface-associated proteins were reported to mediate bacterial adhesion.

L. lactis subsp. *cremoris* IBB477, originally isolated from Polish raw milk, was shown to be tetracycline-resistant and to persist in the GIT of germfree rats. The present work was focused on the adhesive and muco-adhesive properties of *L. lactis* subsp. c*remoris* IBB477. Interactions between IBB477 and bare or mucin-coated polystyrene were probed under dynamic conditions, using the shear stress flow chamber. Then, putative genetic determinants encoding adhesion/muco-adhesion capacity of IBB477 were identified by the sequencing of the strain genome. Based on the bioinformatic analysis of genome sequence, twelve knockout mutants in putative adhesion genes were constructed and tested for their adhesive properties to bare and mucin-coated microtiter plates, in comparison with the wild type strain. Two mutants exhibiting low level of adhesion: Δ*mub* and Δ*prtP* (in genes encoding mucus-binding protein and proteinase, respectively) were chosen for further analysis with the muco-secreting cell line HT29-MTX.

Considering the persistence in the GIT as well as adhesive properties, *L. lactis* subsp. *cremoris* IBB477 was chosen as carrier strain for expression of an influenza hemagglutinin (HA) protein for induction of mucosal immune response.

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O15.4

Multifunctional snake venom phospholipases A_2: promising tools in biotechnology

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Snake venoms are a rich source of pharmacologically active molecules. The secreted phospholipases A_2 ($sPLA_2$) found in *Viperidae* snakes represent a homogenous superfamily of enzymes sharing similar structural scaffolds but exhibiting divers toxic and pharmacological properties. These multifunctional interfacial enzymes catalyze the hydrolysis of phospholipids, but also form high affinity complexes with membrane bound or intracellular neuronal receptors, blood coagulation factors and natural PLA_2 inhibitors, and exhibit other specific biological functions (Faure & Saul, 2012). We focused our study on crotoxin, a potent neurotoxin with PLA_2 activity, from *Crotalus durissus terrificus* venom. We have solved the crystal structure of the heterodimeric crotoxin CACB complex and identified key residues involved in the stability, toxicity and enzymatic activity of this potent β-neurotoxin (Faure *et al.*, 2011).

More recently, using surface plasmon resonance and other biochemical and biophysical techniques, we identified two novel specific targets of rattlesnake PLA_2: the cystic fibrosis transmembrane conductance regulator (CFTR) and bacterial pentameric proton-gated ion channel (GLIC). We investigated the physiological relevance of PLA_2-CFTR and PLA_2-GLIC interactions and our electrophysiological studies enabled us to discover new functions of snake venom PLA_2. We showed that the CB subunit of crotoxin potentiates Cl^- channel current of wild-type and mutated CFTR (DF508, the most frequent mutation) and offers an original perspective to develop novel therapeutic agents against cystic fibrosis.

We also showed that the CB subunit, interacting with full length purified and solubilized pentameric GLIC, regulates proton-gated ion channel activity. Given that the crystal structure of the bacterial homolog GLIC displays a remarkable structural homology with eukaryotic pLGICs (Corringer *et al.*, 2012), GLIC and its ligand may serve as a model for the identification of binding sites and the development of new drugs applicable for human receptors. Indeed, defects in pLGICs lead to a variety of human brain diseases. Co-crystallization trials with the new ligand CB are currently in progress for structural studies of PLA_2-GLIC interactions.

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Posters

P15.1

Structural determination of 5' UTR RNA motifs

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5' untranslated regions of mRNA contain cis-regulatory elements frequently forming secondary structures including IRESes, binding sites for RNA binding proteins, uAUGs and uORFs. These sequences play an important role in translation regulation by promoting or inhibiting translation intiation, affecting mRNA's stability and also acting as riboswitches. As impairment of this regulation machinery perturbs cellular metabolism, leading to various physiological abnormalities studying it at a structural level seems to be an important research subject.
The aim of my research is to determine the structure of 5' UTR RNA motifs using mainly X-ray crystallography method, in combination with low-resolution structural probing methods and theoretical structure prediction. Although X-ray crystallography method for structure determination is widely used for protein structure determination it can be also used for RNA. X-ray crystallography technique employs single crystal X-ray diffraction (SXRD) to unambiguously determine the three dimensional structure of large biological molecules at atomic resolution. The strength of this method lies in the high level of accuracy it provides and lack of the size limitation for the molecules that are analyzed. The steps involved in this technique consist of sample preparation, crystallization, X-ray diffraction and structure determination. But as the surface of RNA molecules is dominated by a poor differentiated regular array of negatively charged phosphates, the crystallization of RNAs remains a formidable experimental challenge which makes low-resolution structural probing methods like SHAPE, CD etc. and theoretical structure prediction also important. Structural insight obtain using different structural characterization methods will help in understanding the different mechanisms of translation regulation, impact of 5' UTR structure on gene expression and linkage between mutations in UTR coding sequence and expression abnormalities.

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P15.2

Lipase from *Rhizomucor variabilis* as useful tool for the biodiesel, ester synthesis and detergent formulation

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Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are widely used in biocatalysis due to their ability to catalyse the hydrolysis of triacylglycerides in aqueous solutions and also synthetic reaction in organic media. Lipases must be reasonably thermostable and active in organic solvents if they are to be used in wide range of synthetic reactions.
Lipases constitute the most important group of biocatalysts for the synthesis of biodiesel and fine chemicals and are also important in the dairy industry, in detergents manufacturing [1, 2].
We assessed the activity and stability of lipase from *Rhizomucor variabilis* in a variety of organic solvents. This enzyme clearly showed stability in high concentrations of certain organic solvents, including methanol and ethanol. Thus we have demonstrated the potential application of this lipase as a catalyst for ester (isoamyl acetate, butyl caprylate and ethyl oleate) and biodiesel production.
Esterification increased steeply with time during early stages of the reaction to attain a major part of the final conversion in 48 h and gradually reached the equilibrium maximum at 72 h, remaining constant thereafter.
Lipase from *R. variabilis* catalyzed biodiesel production in the presence of methanol and rape oil or sunflower oil. The production reached at maximum level at 48 h in the case of rape oil.
Since, the present lipase exhibited temperature and pH kinetics suitable for detergent formulation, detailed characterization of lipase was carried out to evaluate its potential as an additive in detergents. The lipase from *R. variabilis* is stable in commercial detergents. The enzyme retained >76% activity in the presence of commercial detergents like Ariel, Vizir and Bonux. Interestingly, the present lipase is highly stable towards oxidizing agents and was completely stable after 1 h at room temperature in the presence of 1% hydrogen peroxide.
This enzyme could therefore be considered as a satisfactory and promising candidate for further industrial application principally ester and biodiesel production and cleaning process.

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P15.3

First heterologous expression and structural insight into the interferon antagonist protein from Rift Valley Fever Virus

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Rift Valley Fever Virus (RVFV) is a member of the diverse *Bunyaviridae* family of small arthropod-borne viruses, capable of causing life-threatening haemorrhagic fever in animals and humans, leading to dangerous outbreaks and severe economic losses. It is an emerging and zoonotic pathogen, and as such, anthropogenic and environmental factors might soon redistribute the virus from its endemic areas in sub-Saharan Africa and South America to Europe or North America, which is a severe and realistic public health concern. The non-structural (NSs) protein is one of only six proteins encoded by the virus. It is the main virulence factor of RVFV, responsible for shutting down the interferon response in the host cell during the early stage of infection. This is achieved by downregulation of whole-cell transcription through interference with the TFIIH complex assembly, as well as blocking activation of the interferon-β promoter and several other interactions. This and all other NSs proteins from the family *Bunyaviridae* have evaded structural characterization due to poor solubility and stability. Here, we present the methodology for efficient large-scale expression of the stable NSs protein and first insight into its structure and behaviour in solution studied with a range of biophysical methods including NMR spectroscopy and circular dichroism.

P15.4

A novel biocatalytic strategy for the synthesis of benzylisoquinoline alkaloids

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Benzylisoquinoline alkaloids are a complex and diverse group of natural products consisting of more than 2500 known structures, found in the distantly related families including the *Papaveraceae*, *Ranunculaceae*, *Berberidaceae*, *Fumariaceae*. These compounds, naturally involved in the chemical defense of plants against herbivores and pathogens, are also pharmacologically active. Morphine is a natural product with high medicinal significance; codeine is used as an analgetic; berberine and sanguinarine are used as antimicrobials; papaverine and (+)-tubocurarine work as muscle relaxants. The structural complexity of these pharmaceuticals often renders chemical synthesis impractical as an alternative to extraction of the secondary metabolite from cultivated plants for their commercial production.
The enzymatic pathways leading to the amazing diversity of benzylisoquinoline derivatives have been shown to originate from a common route in which the first committed step consists of the Pictet-Spengler condensation of dopamine with 4-hydroxyphenylacetaldehyde to yield the benzylisoquinoline central precursor, (S)-norcoclaurine (higenamine). The enzyme catalyzing this reaction has been identified as norcolaurine synthase (NCS). The protein form *Thalictrum flavum* has been recombinantly expressed, purified and structurally characterized in our lab [1]. We also developed anefficient, stereoselective, green synthesis of (S)-norcoclaurine using the recombinant (S)-norcoclaurine synthase (NCS) enzyme, starting from the cheap tyrosine and dopamine substrates in a one-pot, two steps process [2].
It has been observed that NCS shows a strict requirement for the dopamine while the substrate specificity for the aldehyde substrate is quite relaxed [3]. Anyway, most of the aldehydic substrates are not commercially available and their chemical synthesis is not practical. For this reason we tested in situ production of aldehydes using a plant diamine oxidase (DAO) starting from the corresponding amines.
Preliminary results show that DAO efficiently converts several aliphatic and aromatic ethyl amines. Moreover, NCS is able to catalyze the Pictet-Spengler cyclization of the newly synthetized aldehydes with dopamine, leading to the production of a new class of benzylisoquinoline alkaloids.

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P15.5

An *in vitro* infection of human leukocytes with *Candida albicans* to study of host-pathogen interactions

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A rapid development in medical care, including transplantation procedures, anticancer therapy, extensive use of antibiotics and an emergence of immunodeficiency syndrome contributes significantly to an increased incidence of infectious diseases. An infection represents an acquisition of a microbe by a host and mostly is followed by multiplication of the microbe in or on the host body. Whereas, a disease arise as a manifestation of host tissue damage caused by the microbe, termed as a pathogen of this host [1]. Nevertheless, not every infection results in disease and similarly not every microbe becomes the pathogen for each host. In respect to this, considerable efforts are directed onto understanding the basis of interactions between the mammalian host and pathogens and new approaches, using cell lines or tissue models are applied to investigate this relation [2]. In our studies, we have focused on opportunistic fungus, *Candida albicans*, which is a principal agent of superficial or systemic infections, especially problematic in individuals with host defense system impaired. The aim of this project was to use a human leukemia cell line (HL-60) to develop an *in vitro* fungal infection and to evaluate how the antifungal treatment (*e.g.* Nystatin) affects the fungal-leukocyte interaction. Firstly, an antifungal activity against *Candida albicans* and cytotoxicity on HL-60 cells were examined separately. Subsequently, the performance of Nystatin in the *Candida* infection of human leukemia cell line was analyzed by using an inverted microscope. The extent of leukocytes damage/viability was measured using lactate dehydrogenase activity and live/dead staining. *Candida* viability was also determined by live/dead staining and fluorescence imaging.

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P15.6

Modulation of biological activity of anticancer drug doxorubicin by pentoxifylline

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Direct interactions (i.e. intercalation) between small aromatic ligands and DNA are among the most common mechanisms of action of anticancer antibiotics used in chemotherapy. At the same time there is a group of biologically active aromatic compounds (such as methylxanthines, MTX), which has been shown recently to have a potential to modulate direct interactions between aromatic ligands and DNA. Most probably this phenomenon bases on formation of mixed, stacking complexes between ligand and modulating compound molecules, which diminishes concentration of free, biologically active ligand form. Existing statistical thermodynamical models allow analysis of non-dimerizing aromatic ligands. However, large group of interesting ligands, including anticancer drugs, may form homo-aggregates. Doxorubicin (DOX), which is widely used in cancer treatment, is a well described example of anthracycline aromatic anticancer drug with potential to form homo-aggregates. DOX intercalates to DNA and inhibits topoisomerase II enzyme, thereby blocking the replication process in tumor cells. Unfortunately DOX therapy causes numerous adverse effects, such as: myelosuppression, hyperpigmentation of previously radiated areas, red urine, tissue necrosis and cardiomyopathy. In this work, using UV-Vis spectroscopy and computational methods we demonstrated de-intercalation of DOX molecules from calf thymus DNA in the presence of synthetic MTX - pentoxifylline (PTX). Moreover, to obtain these results, we used newly developed statistical thermodynamical model, which for the first time describes interactions of dimerizing ligands both with DNA and PTX. Based on such analysis we determined every concentration of each mixture component as well as appropriate association constants. Furthermore, to investigate the biological effects of observed interactions, the Ames mutagenicity tests based on *Salmonella typhimurium* TA98 strain were performed. We demonstrated the reduction of DOX mutagenic effects in the presence of PTX molecules, which is closely connected to the concentration of DOX biologically active free form, calculated on the basis of previously obtained DOX-PTX mixed association constant.

P15.7

Potent platelet aggregation inhibitor from Echis multisquamatis snake venom

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Introduction: Platelets are the main cells involved in thrombus formation. Thus inhibition of platelet aggregation may provide an effective approach for prevention of coronary artery thrombosis and rethrombosis in the genesis of acute myocardial infarction. The aim of present work was the purification and characterisation of the biological activity of the platelet aggregation inhibitor from *Echis multisquamatis* snake venom (PAIEM).
Methods: Ion-exchange and size-exclusion chromatography, Laemmli protein SDS-PAGE were used for PAIEM purification and characterisation. Aggregation of platelet-rich plasma (PRP) and washed platelets in the presence of PAIEM was studied on aggregometer Solar-AP2110; changes of shape and granularity of platelets in the presence of PAIEM were studied on flow cytometer COULTER EPICS XL. Indirect ELISA was used for the determination of target of PAIEM on platelet surface.
Results and discussion: 6-kDa protein from *E. multisquamatis* venom was purified and analysed by SDS-PAGE. It was shown to be a potent inhibitor of human platelet aggregation. IC50 of inhibitory effects on ADP-induced aggregation of washed platelets and PRP were determined: 1.58 and 0.75 μM respectively. PAIEM did not change platelet granularity or shape and did not affect thrombin-induced platelet activation measured by flow-cytometry. In the same time ELISA study showed that PAIEM distinctly inhibited the binding of anti-IIb antibody to activated platelets. So purified protein was the antagonist of platelet glycoprotein GPIIb-IIIa.
Conclusions: Novel highly specific inhibitor of platelet aggregation was purified. It was direct antagonist of GPIIb-IIIa platelet receptor that did not cause any changes in platelet shape and granularity during platelet activation. Based on the experiments described above PAIEM have been shown to be potent inhibitor of GPIIb-IIIa mediated platelet aggregation.

P15.8

Flocculation activity of exopolysaccharides isolated from *Actinomycetes*

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Actinomycetes are very heterogeneous group of organisms, widely occurring in different environments. This bacteria are Gram-positive producers of many biologically important substances like antibiotics, enzymes, biofilms, compounds responsible for mycorrhizae and protection against other microorganisms. Additionally, *Actinomycetes* are able to form extracellular polymeric substances called bioflocculants, with flocculation activities. These polymers are used in industry as a potential purifiers of wastewater, heavy metals removals as well as fermentation and downstream processing substances. Nowadays there are many investigates about exploitation new kind of flocculants isolated from different microorganisms like bacteria and fungi, to find the most effective and relatively inexpensive source of flocculating polymers.
In this study, four strains of flocculant-producing microorganisms were investigated and their flocculating activities were tested and compared. During bacterial growth a content of carbohydrates was determined by the phenol-sulfuric acid method and a protein content was measured by Bradford method. Extracellular bioflocculants were extracted from growing medium of each strains using cold ethanol as a precipitant and lyophilized to obtain partially purified products. Based on flocculating activity and mass of vacuum-dried bioflocculants, two bacterial strains were selected for further analysis. Moreover, the surface morphology of bacterial bioflocculant was observed by scanning electron microscope (SEM).

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P15.9

High-resolution and high-speed atomic force microscopy simultaneous to advanced optical microscopy

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In recent years, atomic force microscopy (AFM) has become a well-established technique for single molecule studies and even sub-molecular scale research. Several new developments in terms of faster AFM imaging and imaging modes, based on the phase or frequency, have been established in order to decrease the cantilever response time and increase the AFM's scan speed, e.g., for studying molecular dynamics.
The novel NanoWizard® ULTRA Speed AFM combines the latest scanner technologies and compact design allowing a full integration of AFM into advanced commercially available optical microscopy. Thus, fast AFM imaging of approximately 1 frame per second can be seamlessly combined with methods such as, fluorescence, confocal, TIRF, STED microscopy and many more. Individual molecule dynamics can now be studied with AFM and simultaneously with optical microscopy by applying JPK's tip scanner technology.
With JPK's HyperDrive™ sub-molecular resolution is achieved even on soft samples imaged in liquid environments. It allows for imaging with smallest amplitudes of often approximately 0.2 nm for lowest tip-sample interaction. Topographical images of membrane proteins and DNA-origami are presented. It has been shown that the phase response in phase modulation AFM (PMAFM) is faster allowing higher imaging speeds for the study of molecule kinetics. In conjunction with JPK's NanoWizard® ULTRA Speed AFM, a dynamic biomechanical study of Bacteriorhodopsin (bR) when interacting with photons will be discussed.
More than half a century after the first high-resolution electron microscopy images of collagen type I banding of 67 nm have been reported, now with the NanoWizard® ULTRA Speed AFM we could gain a high-resolution temporal insight into the dynamics of collagen I fibril formation and its characteristic 67 nm banding hallmark. The literature still abounds with conflicting data regarding the models of its fibril formation, structural intermediates, and kinetics. AFM is the only currently available high-resolution imaging technique amongst many to offer insight into the collagen I fibrillogenesis by operating in situ. The described technique could be instrumental for future studies of the structural dynamics of protein systems, etc.
The systems newly gained flexibility will also be demonstrated on a study of living fibroblast cells directly imaged in their culture petri dish at 37 degrees C. Here, the dynamics of individual membrane structures is investigated with AFM while simultaneously observing the individual living cell with optical phase contrast. The unambiguous correlation between AFM and optical microscopy is achieved by the DirectOverlay™ technique.

P15.10

Properities of laccase from Trametes sp. immobilised on ferromagnetic matrix

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Mushrooms of the genus Trametes developed multiple metabolic pathways enabling them to degrade present in plant tissues, polymers of aromatic compounds such as lignin. The mineralization of the polymer involved is complex enzyme complex, one of the main elements is p-diphenol:dioxygen oxidoreductase (EC 1.10.3.2) called laccase. Ability this enzyme to degrade aromatic hydrocarbons and their derivatives has been applied in many industrial process. The purpose of this study was to determine the catalytic properties of laccase immobilized on ferrmagnetic matrix and assess the usefulness of the system for degradation selected synthetic dyes. Laccase was isolated from the liquid culture Trametes sp. obtained form the area of Puszcza Wkrzańska forest. The enzyme was purified by a three-phase partitioning, ion-exchange and molecular sieve chromatography. The purity of enzyme was monitored by SDS-electrophoresis. The purified enzyme was immobilized on ferromagnetic particles, that were modificated by 3-aminopropyltriethoxysilane (APTES) and finally activated by glutaraldehyde. Immobilized laccase showed similar kinetic parameters to the form of the free enzyme retaining its properties with respect to the optimum temperature and pH.There were no significant reduction in the degree of affinity for substrates of the immobilized enzyme as 2,2'-azinobis-3-ethylbenzothiazoline-5-sulfonic acid (ABTS) and 2 - metoxyphenol. This suggest an minimal conformational changes in the enzyme structure and a high efficiency of enzyme immobilization process.
The immobilized enzyme, showed high performance in decolorisation of malachite green and methylene blue. However, the dyes degradation required a presence of a mediator such as ABTS. Obtained results suggested a potential usefulness of the tested system in the purification of industrial waste water containing these dyes.

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P15.11

Spheres based on blend of two functionalized spider silks as selective and stable anticancer drug carriers

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Anticancer agents that are administered systemically at therapeutic doses may cause serious adverse events. One of strategies for cancer treatment is to design a nanodelivery system, directing active agents specifically to the site of tumor, therefore avoiding potential side effects. Bioengineered spider silk is a biomaterial that combines superb mechanical properties, biocompatibility and biodegradability with a good accessibility and simple purification procedure. Thus, spider silk has extensively been explored as a material for numerous biomedical applications. Silk protein can self-assemble into spheres of nano- and micrometrical size. Moreover, genetic engineering enables the functionalization of silk by adding the peptide encoding sequences of the desired attribute. Such functionalized spider silk spheres may serve as a carrier for targeted cancer therapy.
The aim of present study was to obtain novel drug delivery carriers made of two functionalized spider silk proteins and their blends.
The bioengineered silk proteins (MS1 and MS2 based on MaSp1 and MaSp2 proteins from *N. clavipes* spider, respectively) and its Her2-oriented hybrid variants able to target cancer cells were designed. Stable silk particles were obtained by a simple aqueous process triggered by potassium phosphate. The MS1/MS2 blends were produced in different weight ratios. Spheres were characterized in terms of size and morphology (by SEM), zeta potential, secondary structure (FTIR) and stability (spectrophotometry). The anticancer therapeutic doxorubicin (Dox) was incorporated into MS1/MS2 spheres and drug release studies were performed. The binding (by confocal microscopy and flow cytometry) and drug delivery potential (by MTT assay) of silk particles to cancer cells were investigated.
Spheres made of blends of MS1 and MS2 demonstrated differences in morphology, stability, zeta potential and binding to targeted cells comparing with homogenous MS1 spheres. The increasing MS2 concentration into MS1/MS2 blend improved morphology and stability of particles, yet lowered their cell binding efficiency. However, for functionalized MS1/MS2 blends at a weight ratio of 80/20%, the binding efficiency was maintained with improved sphere stability. The release rate of Dox was dependent on pH. The functionalized silk spheres made of MS1/MS2 blend loaded with Dox significantly higher reduced the cell viability of Her2-positive cells comparing with control spheres without the targeting domains and Her2-negative cells.
A blending process can serve as a controlling factor of particle properties. Spheres made of blend of functionalized silks were efficiently loaded with drug and specifically targeted and killed cancer cells. Silk spheres can be used for the delivery of a therapeutic agent to the tumor cells.

P15.12

Isolation of cytochrome c oxidase from different sources

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Cytochrome c oxidase (CcOx), the copper hemoprotein of the inner mitochondrial membrane, requires for its activity phospholipids, is the side of major oxygen utilization and an associated energy coupling for oxidative phosphorylation [1]. Additionally this enzyme shows the pseudocatalase activity [2]. The structures of heme, protein as well lipid components of CcOx in comparison with the mechanism of oxygen reduction in this enzyme are considerably interested [3, 4]. Lately the potential of cytochrome c oxidase in the construction of electrochemical biosensors for cytochrome c, superoxide dismutase and oxygen radicals [5] as well cyanide [6] determinations were described.
The aim of this research was the isolation of the mitochondria from yeast cells and bovine hearts, showing the highest cytochrome c oxidase activities. In the first stage of optimization procedure the screening of different yeast strains and optimization of culture conditions were done. The baker yeast (Lu-com10) growing on lactate medium showed the highest CcOx activity in the isolated mitochondria. The level of cytochrome c oxidase activities in mitochondria was strongly depended from the yeast growing temperature and the level of oxygen in the medium. The optimized procedure gave an high effective method (above 95% living organelles) for mitochondria purification that were essentially free of contamination by other organelles. The isolation of mitochondria from bovine hearts was mainly depends from the quality of the hearts. The best results was obtained on the materials when the hearts was immediately taken in ice from the abattoir after the cattle slaughter. The isolation of mitochondria must began no later that 1 hr after taking it from the animal body. The obtained mitochondria were next used for the isolation of cytochrome *c* oxidase.

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P15.13

Expression of *Saccharomyces cerevisiae ERG20* gene encoding farnesyl pyrophosphate synthase in *Trichoderma atroviride* improves antifungal and biocontrol properties

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In this study we present a new method to obtain the *Trichoderma* strains with enhanced antifungal and biocontrol activities. The method is based on the increase synthesis of the mevalonate pathway products. In this pathway some metabolites are produced such as terpenoids, trichodermin, harzianum A, mycotoxin T2, lignoren, ergokonin A and B and viridin which are known from their antifungal and antibacterial activities. All these compounds are synthesized from farnesyl pyrophosphate (FPP) which is itself synthesized by farnesyl pyrophosphate synthase encoded by *ERG20* gene. FPP is also a substrate for dolichol and ergosterol production which are indirectly engaged in antimicrobial action.
In this study we increased production of FPP in *T. atroviride* by overexpression of the yeast *ERG20* gene. We based on the assumption that the increased activity of FPP synthase would stimulate production of all products of the mevalonate pathway. Five transformants showed higher activity of FFP synthase. Simple screening of the hydrolytic properties of the *ERG20* transformants revealed that they grew faster on plates with polycarbohydrates as carbon sources. Detailed studies showed higher cellulotytic and chitinolytic activity of enzymes secreted to the cultivation medium by the transformed strains.
Antifungal activity was examined by cultivation of plant pathogen *Rhizoctonia solani* in the atmosphere of volatiles liberated by the transformants and the control. We also analyzed growth of *Pythium ultimum* on plates which were previously overgrown by *Trichoderma* strains and were filled with their metabolites. Both experiments showed significantly stronger inhibition of growth of the pathogens by the transformed strains compared to the control. Since the above experiments revealed enhanced antifungal properties of the transformed strains we performed plant tests using the bean *Phaseolus vulgaris L.*. Transformed strains increased both, the germination rate and the size of plants growing in soil infected by *Pythium ultimum* compared to the control strain.
To conclude, an increased activity of the mevalonate pathway caused higher activity of hydrolytic enzymes and increased production of volatiles and secondary metabolites and that way boosted antifungal and biocontrol activities of the *Trichoderma ERG20* transformants.

P15.14

Biochemical characterization of the *Helicobacter pylori* Hp0377-determination of the pK_a values of both cysteines present in the CSYC catalytic motif

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The bacterial proteins of the Dsb family catalyze the formation of disulfide bonds, a post translational modification present in multiple extracytoplasmic proteins that increases the stability of tertiary and quaternary protein structures. However, some proteins, such as apocytochrome *c*, must be present in the periplasm in reduced form. The process of cytochrome *c* maturation (Ccm) requires presence of reduced thiols of the Cys-Xaa-Xaa-Cys-His motif in apocytochrome for ligation with haem. Since the main periplasmic dithiol oxidases, DsbA and DsbA-like proteins, randomly introduce disulfide bonds into apocytochromes, bacteria evolved a redox system able to revert disulfides to reduced cysteine residues in highly oxidizing environment of periplasm. Proteins responsible for this process, CcmG (DsbE), contain a catalytic CXXC motif in the thioredoxin domain. Our experimental results suggest that *H. pylori* Hp0377 acts similarly to CcmG in the cytochrome maturation process.
Hp0377 has a redox potential of -171 mV. The value is close to that of most CcmGs and indicates that the protein is a weak reducing agent. An important characteristic of thiol-disulfide oxidoreductases is the decreased pKa value of the reactive cysteine, what determines high reactivity in thiol-disulfide exchange reactions. Previous studies on several Dsb proteins have revealed a strong relationship between pK_a values of the active-site cysteine residues and their redox properties, such that the lower the pK_a value of the N-terminal cysteine residue, the higher the reduction potential. Initial experiments suggest that Hp0377 is not a typical CcmG protein. The pK_a of the nucleophilic N-terminal cysteine of Hp0377 CSYC motif is equal to 3.49±0.29. The value is comparable to the pK_a of EcDsbA oxidase, and it is unusual for CcmGs (for *E. coli* CcmG pK_a=6.8; for *B. subtilis* ResA pK_a=8.8). Because of the unique Hp0377 pK_a value, we decided to introduce point mutations into the catalytic motif CSYC. Point mutations changing C89 and C91 into serines were introduced by standard methodology and confirmed by sequencing. Recombinant modified Hp0377s were purified by affinity chromatography and will be used to measure pK_a of both cysteines what will enable precise measurement of pK_a of each of the amino acids free of interference from the second cysteine in the catalytic motif.

P15.15

Single walled carbon nanotubes and congo red for delivery of doxorubicin to cancer cells

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Most of the present anti-cancer drugs have low efficiency and unexpected side effects. Thus drug delivery systems that combine targeted delivery and controlled release without damaging the healthy tissue, have been widely investigated in the past several decades. The carrier should show high drug-loading capacity and low toxicity, ensure the effective transport of the carried drugs across cell membranes and be easily removed from the body. The model drug used in this study is doxorubicin (DOX) - the commonly used chemotherapeutic agent. Single walled carbon nanotubes (SWNTs) are studied as potential transporters of drugs absorbed to their surface. The supramolecular ribbon-like assemblies created by the dyes of the Congo red (CR) type can incorporate certain drugs (especially polycyclic, planar molecules like DOX) and thus can also be considered as potential drug carriers. The combination of both systems appears to be a promising solution. The aim of the study is to determine whether it is possible to use Congo red-functionalized carbon nanotubes for drug delivery into cells, and whether CR improves drug-loading properties of SWNTs.
The method of dissolution of SWNTs by CR and binding of DOX was developed. AFM and TEM analysis of the SWNTs-CR complexes showed the increase in the diameter of the SWNTs, as compared to the control sample, suggesting that the dye binds the nanotube in its supramolecular form. SWNTs covered with CR and DOX showed the increased rigidity, thus straight and shortened (broken) SWNTs-CR-DOX complexes were observed in AFM. This may be of practical importance, since shorter nanotubes can better permeate the membranes and are more easily excreted. The quantitative analysis of the amount of CR complexed to SWNTs was performed. The release of DOX from these complexes was studied in different pH conditions. The highest rate of DOX release was observed in acidic solutions. This is advantageous since the pH of the endosomes and the tumor is low.
Studies concerning the cytotoxicity of the above described system were performed using the human glioblastoma U87MG (doxorubicin sensitive) and human fibroblast Hs27 cell lines. The addition of the SWNTs-CR-DOX led to the inhibition of cell proliferation, while cells treated with carriers free of DOX (CR, CNT-CR) showed the proliferation at the level of the control cells.
Congo red can be directly adsorbed onto the surface of SWNTs to afford a water soluble and biocompatible drug delivery system. The results indicate that this type of carrier potentially can improve the pharmacological efficiency and reduce potential side effects.

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P15.16

Impaired dolichol formation affects cell wall biogenesis and filamentous growth of *Candida albicans*

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Glycosylation of the secreted and plasma membrane proteins is an essential and abundant protein modification in all eucaryotic cells. Dolichyl-phosphate acts as a glycan carrier during the biosynthesis of the lipid-linked oligosaccharide in N-linked protein glycosylation. Dolichyl-phosphate-mannose serves as monosaccharide donor in the later steps of the N-linked glycosylation, in O-mannosylation and for the GPI-anchor synthesis. De novo dolichol is synthesized in the mevalonate pathway. Cis-prenyltransferases (Rer2p and Srt1p) are involved in succesive condensations of isoprenyl pyrophosphate (IPP) units to farnesyl pyrophosphate (FPP) resulting in polyprenyl pyrophosphate (PolPP). During the next steps of reaction PolPP is dephosphorylated and reduced to form dolichol. Only phosphorylated dolichol enters the glycosylation pathway.
It was previously found (Cantagrel *et al.*, 2010) that human SRD5A3 (steroid 5α-reductase type 3) gene is responsible for converting of polyprenols to dolichols and mutations in this gene are the basis of the new type of congenital disorders of glycosylation (CDGs). We therefore asked whether *C. albicans* orf.19.209, the ortholog of hSRD53 and *S. cerevisiae DFG10* plays a role in dolichol formation and have constructed *Cadfg10Delta* null mutant using the URA-blaster protocol. Over 70% decrease in polyprenol to dolichol conversion in the *Cadfg10Delta/Cadfg10Delta* null mutant unequivocally indicates that *Ca*Dfg10p is involved in the reduction step in the dolichol biosynthetic pathway. Decreased level of dolichol synthesis reflected defects in glycosylation of well characterized glycoproteins: soluble N-acetylglucosamindase and membrane bound Phr1p. Moreover, an increase in chitin content in the *Cadfg10Delta* null mutant indicated alterations in the cell wall assembly. However, elimination of Dfg10 protein rendered strain still synthesizing dolichol (26% of the wild-type). *C. albicans* orf.19.3293 is the ortholog of *S. cerevisiae TSC13* gene, encoding the only other enzyme with the predicted steroid 5α-reductase domain involved in very long chain fatty acid (VLCFA) elongation. We therefore have constructed *Catsc13Delta* mutant disrupted in one *TSC13* allele in the parental strain (CAI4) and the *Cadfg10Delta* null mutant. Numerous attempts to obtain *Catsc13* homozygous strain failed what indicates that *TSC13* is an essential gene. HPLC analysis of polyisoprenoid family showed 53% decrease in dolichol level in *CaTS13/Catsc13Delta* and further drop in dolichol content (24% of control) with the concomitant 59% increase of polyprenol to dolichol ratio in the *Cadfg10Delta/Cadfg10Delta CaTSC13/Catsc13Delta* cells. These results point out that in the yeast *S.cerevisiae* and pathogenic *C.albicans* dolichol level is controlled by at least two proteins Dfg10 and Tsc13.
The activity *in vitro* of GlcNAc-transferases (Alg7p, Alg13p and Alg14p) involved in the initial steps of highly conserved dolichol-linked saccharide formation (Averbeck *et al.*, 2007) and DolPMan synthase responsible also for O-mannosylation and GPI anchor synthesis is reduced in synthetic manner in *Cadfg10Delta/Cadfg10DeltaCaTSC13/Catsc13Delta*

indicating that disturbed Tsc13p function in combination with the deletion of *DFG10* my affect the early steps in protein glycosylation pathway. *Cis*-prenyltransferase activity determined *in vitro* was significantly lower in microsomal membranes isolated from *CaTSC13/Catsc13Delta* and *Cadfg10Delta/Cadfg10DeltaCaTSC13/Catsc13Delta* cells reflecting decreased dolichol level *in vivo* in both strains. Disruption of the *TSC13* allele in *Cadfg10Delta* background rendered the mutant strain very sensitive to agents known to interfere with cel wall biogenesis such as CFW and Congo Red. On the other hand, in the presence of cancidas *Cadfg10Delta/Cadfg10DeltaCaTSC13/Catsc13Delta* were more resistant to this agent than *CaTSC13/Catsc13Delta* and *Cadfg10Delta/Cadfg10Delta* suggesting decreased level of β-1,3- glucan polysaccharides. Moreover, the single *CaTSC13* gene deletion in the *Cadfg10Delta* null strain resulted in reduced hyphae formation in the presence of 10% serum while on Spider medium the mutant failed to induce filamentous growth, considered to be necessary for virulence.

Our studies indicate that the block in the reduction step of dolichol formation affects the cell wall integrity and morphological transition of *C.albicans* under hyphae inducing conditions and can be considered as a target for the development of new therapeutical strategies against this important human pathogen.

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P15.17

The role of chaperon DnaK in biosynthesis of nanocellulose by *Gluconacetobacter xylinus*

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Cellulose synthesized by bacteria of the genus *Ga. xylinus* has unique properties such as high crystallinity degree, high purity and water folding capacity, therefore it is widely used in the food, electronics and chemical industry as well as in medicine [1]. Increased use of cellulose and its derivatives in various industries causes that work on improving efficiency and reducing the cost of the polymer synthesis and modification of its properties are the subjects of intensive research. Since the knowledge in this field is still insufficient, it is of high importance both for industrial and scientific purposes to do the research in the understanding of molecular mechanisms of biosynthesis of cellulose.
On the basis of the ITB TUL transcriptome analyzes differentiating the cells able to produce cellulose (Cel+) from those non-producing ones (Cel-) the DnaK chaperone was identified as having a potential effect on the production of nanocellulose by *Ga. xylinus*. Based on literature data it is known, that DnaK plays important role in bacterial stress tolerance, assists a wide range of folding processes, including the folding and assembling the newly synthesized proteins, refolding of misfolded and aggregated proteins, and control of the activity of regulatory proteins or biofilm formation by microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas putida* [2, 3]. A study concerning the role of DnaK in cellulose synthesis by *Ga. xylinus* has not been reported up to date in literature. It is only known that disruption of the equivalent of *dnaK* resulted in a Cel- phenotype. The authors hypothesized that the DnaK is needed for proper folding of establishing the cellulose synthase complex in the membrane, or for transport of such proteins [4].
The aim of current investigation is to analyze the relation between previously selected gene *dnaK* and the biosynthesis of cellulose. The research aimed at obtaining *Ga. xylinus* mutants with *dnaK* overexpression, with *dnaK* disruption and complementation mutants as well as analyse the cellulose membranes synthesis by mutants.

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P15.18

Caveats of biological data analysis (or why you need a bioinformatician)

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Next generation sequencing (NGS) technologies are currently revolutionizing the way we conduct biological research. The advent of cheap, high–throughput sequencing has provided the researchers with efficient tools for such applications as whole genome sequencing or transcriptome profiling, ushering the age of big data in life sciences. Initial problems with handling the vast amount of data generated by sequencing machines were soon overcome by the development of novel algorithms to map and assemble short reads. While the arms race between sequencing throughput and software is far from over, for most applications there is a multitude of computational tools, often able to process the NGS data even on desktop computers. As a result, researchers are no longer forced to concentrate on technical details and can focus on proper experiment design and data analysis.
In our talk we will focus on caveats and good practices in NGS data analysis, particularly in the context of RNA-Seq technology and assembling bacterial genomes. We will raise such subjects as required number of biological replicates and sequence coverage, testing differential expression and preparing libraries for genome sequencing. We will also present the short overview of our solutions for data processing and visualization.

P15.19

Studying of bacterial growth and adaptation in microdroplet chemostat

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Introduction: The introduction of the chemostat[1] was a milestone in the field of microbiology. The primary drawback of conventional technique is a design that places significant constraints on experimental throughput. Droplet microfluidics[2] offers a convenient approach for operating parallel chemostats at a minimal cost of infrastructure, complexity and reagents by compartmentalizing cells and nutrients in plugs of liquid.
Objective: The aim of this project was to provide an automated droplet-based microfluidic technology for highly parallel, long-term studies of microbial physiology, evolution and adaptation.
Methods: The automated system consists of several regions where operations are performed — that we refer to as 'segments' — for: 1) on-demand generation of microdroplets containing cells, reagents, and soluble growth factors; 2) cycling microdroplet chemostats during growth and monitoring of bacteria; and 3) splitting and fusing microdroplets to exchange media and manipulate the concentration of chemical factors in time in each of the microdroplet chemostats.
Results: We were able to perform experiments that require long-term cell growth. The time-dependent concentration of cells followed the Monod's growth curve, characteristic for classical chemostats. The system enables the user to control the composition of microchemostats in time and makes it possible to perform experiments that test the response and adaptation of populations of microorganisms to stress and fluctuating environments. To explore this area, we studied the response of a population of *E. coli* cells to the concentration of chloramphenicol.
Conclusion: We demonstrated a microdroplet chemostat system[3] that enables long-term, parallel monitoring of cell growth in response to extracellular stress. To the best of our knowledge this is the first demonstration of a fully automated droplet microfluidic technology that offers scalability of the number of microchemostats with limitations rooting only in the size of the chip and frequency of operation on droplets, but not in the complexity of the experimental setup.

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P15.20

G-rich oligomers influencing Dicer activity

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One of the most exciting findings of the last two decades in RNA biology is the discovery of small non-coding RNAs that affect gene expression through the RNA interference (RNAi) mechanism. In human, a major class of RNAs involved in RNAi are microRNAs (miRNAs). Up till now over 1500 miRNAs encoded in the human genome have been described. It is estimated that they affect expression of more than half of all our genes. Thus, they play a pivotal role in the regulation of various biological processes, including cell cycle progression and proliferation, differentiation, cell survival and development. Moreover, the misregulation of miRNAs is observed in a wide range of human diseases, including cancer. Therefore, the cellular levels of miRNAs and other RNAi pathway's components have to be tightly controlled both spatially and temporally.

In our studies we focus on the final stage of miRNA biogenesis, i.e. the cleavage of miRNA precursors (pre-miRNAs) by human ribonuclease Dicer to yield ~22-nt RNA duplexes. Although the mechanism of Dicer action is quite well characterized, our knowledge of its regulation is still limited. So far, several protein suppressors or activators of Dicer functioning have been described, however little is known about non-protein factors affecting this enzyme. In our studies we show that apart typical Dicer substrates, also short oligonucleotides may influence Dicer activity through direct interactions with this enzyme, and that this effect may depend on the ternary structure of the oligomers. We demonstrate that *in vitro*, specific G-rich oligomers form complexes with recombinant human Dicer and affect its ability to generate functional miRNAs, acting as universal inhibitors of this enzyme.

Recently it was demonstrated that certain viral RNAs directly bind to Dicer and inhibit its function. Also, endogenous semi-microRNAs derived from mature miRNAs and affecting their role were identified. Whether short endogenous RNA species play a role in miRNA biogenesis regulation by influencing Dicer activity remains elusive, and needs further studies. Nevertheless our data indicate that such mechanism is possible *in vitro*, which may suggest the existence of yet another cellular mechanism orchestrating miRNA and Dicer regulation.

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P15.21

Expression and immunoreactivity of the molecular chaperone ClpB from the bacterium *Leptospira interrogans*, the causative agent of leptospirosis

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Bacterial ClpB is a member of the Hsp100/Clp subfamily of the AAA+ ATPases that solubilizes and reactivates stress-aggregated proteins in cooperation with the DnaK chaperone system. The mechanism of protein disaggregation mediated by ClpB is linked to translocation of substrates through the narrow central channel within the hexameric ring structure of ClpB. The data accumulated over the last few years regarding several bacterial pathogens suggest that ClpB chaperone may play an important role in their virulence. However, currently it is not clear what specific role ClpB plays during bacterial infection. This chaperone can function either as a true virulence factor directly involved in causing disease or as a virulence-associated protein that can be essential to enable the pathogen to colonize its host. We are interested in this aspect of the function of ClpB. We focused on ClpB from bacterium *Leptospira interrogans* ($ClpB_{Li}$), a spirochete capable of causing a disease known as leptospirosis. In humans the disease varies from an asymptomatic flu-like illness to an acute life-threatening infection. Sources of this pathogen are mostly wild (rodents) or domestic animals. Human are infected directly through exposure to urine or water contaminated with urine of mammalian reservoir hosts. More than 1 million cases of severe leptospirosis are reported each year, with a mortality rate of 5% to 20%. Leptospirosis is also a serious economic problem, because it causes abortions, stillbirths, infertility, failure to thrive, reduced milk production, and death in domestic animals such as cows, pigs, sheep, goats, horses and dogs. Despite its severity and global importance, the molecular mechanisms of leptospiral pathogenesis remain still unknown.

The purpose of this study was to clone and express of *clpB*$_{Li}$ in *E. coli* cells, and assess immunoreactivity of its product, the $ClpB_{Li}$ protein. $ClpB_{Li}$ consists of 860 amino acid residues. Sequence alignment of $ClpB_{Li}$ and $ClpB_{Ecoli}$ using the Clustal software shows that the total sequence identity between them is only 52% (27.7% within ND, 45.3% within MD, 72% within NBD1, and 65.7% within NBD2). We found that $ClpB_{Li}$ did not rescue *E. coli DclpB* mutant under heat shock. The lack of complementation effect may confirm species-specificity of ClpB proteins and also suggests that protein substrates recognized by the molecular chaperone $ClpB_{Li}$ differ from proteins aggregated upon heat shock in *E. coli* cells. Furthermore, we found that $ClpB_{Li}$ is able to activate the host immune system, thus suggesting its involvement in pathogenesis of leptospirosis.

P15.22

Biotechnological potential of yeasts isolated from Antarctic environments

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Psychrophilic yeasts are a diverse group of eukaryotic microorganisms characterized by a variety of nutritional preferences and ability to survive at extreme environmental conditions, differentiated both physically and geochemically. It is believed that yeasts have better adaptive potential than bacteria when it comes to cold environments. Along with other psychrophiles, they play essential role in organic matter decomposition and biogeochemical circulation, providing necessary compounds for higher organisms. Huge biotechnological potential is owned to their ability of active growth and metabolism at low temperatures. They manage to accumulate large amount of lipids intracellulary, degrade petroleum hydrocarbons and phenolic compounds, while being a source of cold-adapted enzymes and various types of exopolysaccharides they may be used in pharma and food industry.

Unique properties of enzymatic system of those microorganisms, resulting from specific life requirements, make them an interesting object of study. Consequently, their industrial usage has been recently significantly increased as their usefulness is undoubtful. Their application can reduce energy costs of technological process and allow for selective inactivation of thermolabile enzyme. Furthermore, low temperature of process prevent from bacterial infection, resulting in higher quality of final product.

The aim of presented study was to determine the ability of psychrotolerant yeast to produce adapted to cold biotechnologically interesting enzymes, including amylases, pectinases, cellulases, xylanases, chitinases, chitosanases, β-galactosidases, phytases, lipases and proteinases. Screening was performed for 18 yeast strains isolated from Antarctic soil samples and deposited in the Pure Culture Collection IBT TUL, which were identified by molecular methods as belonging to 8 species: *Cryptococcus gastricus*, *Cr. gilvescens*, *Cr. saitoi*, *Cryptococcus* sp., *Rhodotorula mucilaginosa*, *Debaryomyces hansenii*, *D. macquariensis* and *Candida davisiana*. Yeasts were grown in media supplemented with substrates or inductors of biosynthesis suitable for desired enzymes. The most common was activity of lipases and proteases, while xylanases and cellulases production was not observed in plate tests.

An attractive feature is also their halophilic character. Most of isolates were able to grow in YPG medium with addition of 10 % NaCl. Strains of species *Cr. gilvescens* and *D. hansenii* remained active in medium containing 20 % NaCl.

P15.23

MS2 and EMS2 silks as example of protein bioengineering to modify the affinity for drugs

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The properties like biocompatibility, biodegradability and exquisite mechanical strength made silk an ideal material for wide biomedical applications. Various morphological forms, such as films, fibers or scaffolds, can be easily obtained from bioengineered silk. Silk can also be processed into micro- and nanospheres which may serve as drug carriers.

Two bioengineered silks were constructed: MS2 and EMS2. The sequences of both proteins were based on the consensus motif of MaSp2 spidroin from *Nephilla clavipes* spider. MS2 was obtained by ligation of 15 repeat units, while 15 times repeated unit of EMS2 was modified by adding the glutamic acid. Bioengineered proteins were produced in *E.coli* expression system and then purified by thermal denaturation method. To prepare silk nanospheres the proteins solutions were mixed with potassium phosphate at different conditions depending on 1) concentration of potassium phosphate, 2) pH of potassium phosphate buffer, 3) concentration of silk proteins. Scanning electron microscopy was used to determine the size of spheres and characterize their morphology. Zeta potential was measured using Zetasizer analyzer. The cytotoxicity of spheres was investigated by MTT assay. Two drugs — mitoxantrone and etoposide were loaded into silk particles during the process of spheres formation. Their loading and release profile was measured spectrophotometricaly.

Bioengineered silks — MS2 and EMS2 were constructed, produced and purified. Bioengineered silks formed spheres in conditions above a critical potassium phosphate concentration — 1M for MS2 and 2M for EMS2. SEM analysis revealed differences in spheres size and morphology what was depended on type and concentration of used protein and the pH of potassium phosphate buffer. Zeta potential of spheres made of MS2 was between -1.8 and -16.6 mV while for EMS2 was from -17 to -20.3 mV depending on the preparation variant. The cytotoxicity analysis demonstrated that spheres from both proteins were not toxic. MS2 spheres showed stronger affinity to mitoxantrone while EMS2 spheres had better etoposide uptake. Etoposide demonstrated a pH-dependent release profile.

Both, the type of the protein and the conditions of the spheres formation determine the properties of silk spheres. MS2 and EMS2 particles represent different affinity to anticancer drugs. By modifying the silk sequence, the affinity to drugs can be controlled.

P15.24

Study of trimethine cyanine dyes with different N,N'-substitutions as amyloid-sensitive probes

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The study of the uncontrolled protein aggregation, particularly amyloid fibril formation is among the actual biomedical goals because of the connection of this process to pathogenesis of neurodegenerative diseases (Alzheimer's, Parkinson's diseases, etc.) and series of other harmful disorders. Thus there is a request for analytical tools allowing detection of amyloid fibrils and investigation of the fibril formation process.

Amyloid-sensitive fluorescent dye Thioflavine T is widely used in the works connected with study of the protein aggregation; recently fluorescent cyanine dyes were proposed as specific efficient probes for this application.

Here we report the study of the series of benzothiazole trimethine cyanines with the N,N'-substituents of different nature as probes for quantification of amyloid fibrils and monitoring of the fibrilization reaction using insulin and lysozyme as model proteins.

Studied dyes are virtually non-fluorescent when unbound or in the presence of monomer proteins, but upon addition of fibrillar proteins these dyes increase the emission intensity up to 70 times. It was shown that the nature of substituents could noticeably affect the selectivity of the dye molecules to the fibrils formed by certain protein. The dyes containing alkyl groups or phenyl alkyl groups are more sensitive to lysozyme fibrils, whereas the cyanines with quaternary amino group give more pronounced response on insulin fibrils. The cyanines modified with sulfo-alkyl group, hydroxy-alkyl groups possess the same sensitivity to both fibrillar proteins. The specificity of dyes to different fibrillar proteins could be explained by interaction between the N,N'-substituents of dye with side residues of amino acid chains.

The range of fibrillar insulin quantification by cyanines exceeds that of the commonly used probe Thioflavine T in several times: e.g. the dye containing quaternary amino groups allows determination in the range 0.8–300 mkg/ml that is much wider than for Thioflavine T(5–40 mkg/ml). Besides, the sensitivity of dyes with alkyl groups or phenyl alkyl groups to fibrillar lysozyme significantly overpasses that of Thioflavine T. It was shown that the studied dyes could be used for monitoring the passing of fibril formation reaction of both proteins giving the high reproducibility of results.

Thus we propose these new cyanines as sensitive probes for quantitative detection of fibrillar protein, efficient tools for the study protein aggregation reaction and search of the potential inhibitors of this reaction.

P15.25

RNA oligomers as specific regulators of individual microRNA biogenesis

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Small regulatory RNAs (srRNAs) play an essential role in many biological processes including developmental timing, growth control, differentiation and apoptosis. In humans, the vast majority of srRNAs belongs to a microRNA (miRNA) family. Up till now over 1500 miRNAs encoded in the human genome have been described. It has been estimated that miRNAs control the expression of over 60% of human protein-coding genes. Moreover,it has also been documented that in mammals miRNAs play a very important role in host-virus interactions.

The precise regulation of miRNA biogenesis seems to be of the greatest importance for the functioning of all eukaryotic organisms. Even small changes in the level of individual miRNA accumulation can initiate pathological processes, including carcinogenesis. In our studies we focus on ribonuclease Dicer, the enzyme that excises 19-23 nt miRNAs from 50-70 nt long stem-loop precursors called pre-miRNAs. We hypothesized that RNA molecules can function not only as Dicer substrates but also as its regulators. To verify the above presumption we used the SELEX method to identify RNA oligomers that bind human Dicer. We demonstrated that some of the selected oligomers affected Dicer's ability to generate functional miRNAs. We also found that among the identified RNAs there were oligomers which operated as specific inhibitors. The latter decreased the formation of one miRNA, whereas production of the other miRNAs was hardly influenced. More detailed studies revealed that the selected RNA oligomers can simultaneously bind both Dicer and pre-miRNA. This new class of bifunctional riboregulators interferes with miRNA maturation by interacting with specific pre-miRNAs and by sequestering Dicer. Furthermore, the results of our studies suggest that the mutual interactions between the miRNA precursors and other small RNAs can form a very complex regulatory network which controls miRNA biogenesis and afterwards gene expression.

One of the most challenging issues in the biomedical field is the design and production of highly selective therapeutics targeting particular enzymes or nucleic acid molecules. Our data indicate that one can modulate the production of individual miRNAs by using specific RNA oligomers. The application of such RNA oligomers might be useful for the treatment of many diseases associated with the deregulation of miRNA levels, like cancers, neurodegenerative or immune system, or rheumatic disorders.

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P15.26

Complete genome sequence of *Escherichia coli* BL21(DE3) lytic T4-like bacteriophage Lw1

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Bacteriophages are essential components in microbial communities and play a global role in the transfer and storage of genetic information. While having a practical importance in medicine and agriculture, they can be harmful for industries based on microbial biotechnology and fermentation. Phages constantly cause the lysis of bacterial cells and they can inhibit technological processes leading to dramatic financial losses. *Escherichia coli* BL21(DE3) is at the base of a commonly-used recombinant protein production system. Here we present the complete genome sequence of a new lytic bacteriophage Lw1 isolated from lysed cultures of *E. coli* BL21(DE3) that was propagated to obtain a recombinant protein in a lab bioreactor. Ion-exchange chromatography on DEAE cellulose and CsCl-gradient ultracentrifugation were applied for virus particle separation, concentration and purification.
Electron-microscopic analysis of the phage samples stained with uranyl acetate (2%) showed that Lw1 belongs to the Myoviridae family, morphotype A2. The average phage Lw1 head size is 107x80 nm and tail length is 117 nm.
The virion DNA was obtained using the SDS-phenol extraction method. Sequencing was performed using the 454 pyrosequencing approach. The phage genome contig was assembled with Newbler v2.5.3 that also confirmed its circular genetic map.
The Lw1 virion contains linear dsDNA of 176,227 bp with circular permutation. There are many Dam and Dcm sites completely or partially methylated. GC content is 43.5% and the genome contains 274 predicted genes: 273 protein-coding ORFs and 1 Met-tRNA gene. 150 ORFs (55%) have unknown functions. The gene density is 1.56 genes/kb and the coding density is 95.5%. Average gene size is 618 nucleotides; average protein size is 205 aa. The closest relative phages — RB43, GAP161, RB16, KP27 and KP15 — share with Lw1 91.2% (249/273), 90.5% (247/273), 89.7% (245/273), 86.1% (235/273) and 83.2% (227/273) of homologs, respectively. Thus, phage Lw1 belongs to the RB43-group of pseudo T-even bacteriophages. Both experimental data and bioinformatic analysis showed that Lw1 is a lytic phage and is not able to lysogenize the host cells. No bacterial exotoxin homologs were found in the phage genome. This suggests that bacteriophage Lw1 cannot be considered a human threat in the biotechnological industry.
This genome sequence has been deposited in GenBank with accession number KC801932. Described in this paper is the second version, KC801932.2.

Reference:

Kushkina AI *et al* (2013) *Genome Announc*. doi:10.1128/genomeA00743-13.

P15.27

Sequence-specific inhibition of bacterial translation and growth by a peptide nucleic acid oligomer targeting Helix 69 of 23S ribosomal RNA

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Antisense technology involves targeting or modifying gene expression in a sequence-dependent manner and has been applied to distinguish bacterial species in diagnostic applications or to design antibacterials. The development of synthetic nucleic acid analogues (e.g. locked nucleic acids, peptide nucleic acids) has contributed to the efficient use of the antisense technology [1]. Peptide nucleic acids are synthetic analogues of DNA with a pseudo-peptide backbone containing nucleobases [2]. PNA oligomers have been tested as potential antibacterials [4]. Unfortunately, PNA oligomers are larger than conventional antibacterials and are not easily taken up by bacteria.The most efficient strategy for the delivery of PNA to the microbial cell appears to be the conjugation of PNA with cell-penetrating peptides [4].
The aim of this study was to inhibit protein synthesis and bacterial growth by short PNA sequences targeting 23S ribosomal RNA of Helix 69. Helix 69 is a highly conserved stem-loop in domain IV of 23S rRNA in bacterial 50S subunit and it is a part of the B2a bridge joining the ribosomal subunits [3]. First, we verified sequence-specific hybridization of these PNAs with isolated Helix 69 from *Escherichia coli* and Helix 69 with a sequence corresponding to a human one. Thermal melting experiments and the non-denaturing electrophoresis showed the formation of an RNA/PNA duplex with the *E. coli* Helix 69. No complementary binding to the human Helix 69 RNA loop containing three mismatches was detected. This result proves high specificity and affinity of PNA binding to this helix. For PNA targeted to bacterial Helix 69 we also determined the IC_{50} values (i.e., inhibitory concentration of a substance needed to reduce the synthesis of a reporter protein by 50%) in a cell-free translation/transcription system and MIC (i.e., minimal inhibitory concentration of a substance needed to inhibit bacterial growth after overnight exposure) for bacterial cultures. To assure its entry to bacterial cells, PNA was conjugated to a cell-penetrating peptide. We have shown that PNA targeted to Helix 69 inhibits bacterial translation *in vitro* and bacterial growth.

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P15.28

Interactions of hydrophobically modified polyelectrolytes with liposomes and human cells

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Introduction: Interactions between synthetic polycations with phospholipid bilayers play an important role in several biophysical applications such as gene and drug delivery into cells or antibacterial usage.
Aim: The aim of our work was to study the impact of hydrophobically modified poly(allylamine hydrochloride) (PAH) on a zwitterionic bilayer used as a model of the cell membrane and then on human skin fibroblasts (HSFs).
Methods: Release of calcein entrapped inside liposomes; cells (HSFs); tested chemical compounds: PAH and its cationic, amphiphilic derivatives; viability assay; proliferation assay; immunocytochemical staining; membrane permeabilization: microscopic observation and supernatant analysis; adhesiveness assays.
Results: We investigated the influence of PAH and its amphiphilic derivatives on liposomes and human cells. The results show that the degree of calcein release strictly depends on the presence of hydrophobic domains in the polymer structure. Later the effect on HSFs was tested. While PAH exhibited significant cytotoxic effect, all the derivatives were tolerated much better. We also demonstrated that the treated cells proliferated faster than the control ones, however, this phenomenon was stronger in lower concentrations. Immunostaining revealed changes in the actin cytoskeleton and significant alterations in vinculin distribution after incubation with the polycations. Also adhesiveness tests showed an altered interaction with the substrate. Finally, PAH derivatives seriously increased cell membrane permeability.
Conclusion: The results of our study validate improved biological properties of the synthetized PAH derivatives. Moreover, our research indicates their possible applications in procedures requiring cell membrane permeabilization. In addition, the observed changes in focal contacts suggest that there may be new areas of the PAH derivatives' influence that can be investigated.

P15.29

Synthesis of lipid linked oligosaccharide (LLO), and its role for *C. albicans* morphogenesis

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LLO (DolPPGlcNAc2Man9Glc3) acts as the oligosaccharide donor in protein N-glycosylation, in the lumen of endoplasmic reticulum (ER). It is formed by subsequent addition of sugar residues from the respective sugar nucleotides to the isoprenoid lipid, dolichyl phosphate (DolP). In the final step the oligosaccharide is transferred to the acceptor protein by the oligosaccharyl transferase, which is the protein complex, made up from the nine subunits. The first reaction in LLO assembly is catalyzed by GlcNAc-1-phosphate transferase Alg7p, which produces GlcNac-PP-Dol by adding GlcNac-1P from UDP-GlcNac to DolP. The addition of the second GlcNAc and subsequent formation of $GlcNac_2$-PP-Dol is catalyzed by Alg13p/Alg14p UDP-GlcNAc-transferase with catalytic and membrane anchoring domains are localized on the separate polypeptides (Bickel *et al.*, 2005). Alg7p, Alg13p and Alg14p proteins form multienzyme complex, what allows the channeling of LLO intermediates between active sites without diffusion (Noffz *et al.*, 2009). Based on the results from *S. cerevisiae*, we can predict, that *ALG7*, *ALG13* and *ALG14* genes are essential for N-glycosylation and their mutations may have serious consequences for thephysiology of the microorganisms. Therefore, in *Candida albicans* they might encode proteins standing for the therapeutic targets against the pathogen.
To this end, we have constructed *alg13Δ*/TRp-*ALG13* conditional mutant. GlcNAc-transferase complex activity, determined *in vitro* in the membranes from *alg13Δ*/TRp-*ALG13* cells cultivated in the absence of repressor, was found to be decreased to 52% compared to the wild type. Whereas, under the repressive conditions (40 μg/ml doxycycline), the activity was lower by 12% than the wild type one.
On the other hand, numerous attempts of *alg7* and *alg14* conditional mutants construction were unsuccessful, suggesting essentiality of the *ALG7* and *AlG14* genes.
Moreover, we have determined the activity of DolPMan synthase, which is also involved in O-mannosylation process and GPI-anchor synthesis. In the absence of repressor we observed the reduced activity by 35% compared with the wild type. However, under the repressive conditions of growth, the activity increased to the level of the wild type strain CAI4.
The above results suggest that the first step in LLO assembly is tightly controlled.
The *alg13Δ*/TRp-*ALG13* mutant displayed a growth phenotype in the presence of agents such as Calcofluor White, Congo Red, Caspofungin and Hygromycin B, known to interfere with cell wall biogenesis. The mutant failed to induce filamentous growth on Spider medium and 20% YP serum at 37°C. Furthermore, biofilm formation in the *alg13Δ*/TRp-*ALG13* mutant strain appeared to be severely affected in comparison to the wild type (CAI4) control.
Altogether, the data suggest that suppression of GlcNAc-transferase activity renders strain sensitive to untifungal drugs, suggesting that early steps of N-glycosylation are crucial for maintaining correct cell wall properties and fungus morphological differentiation of *Candida albicans*. The

results of above studies might be exploited in terms of antifungal therapy.

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P15.30

Immunological response of sheep to injections of recombinant SAG1, SAG2, GRA1 *Toxoplasma gondii* proteins coupled to the non-toxic microparticle MDP

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Introduction: Toxoplasmosis, caused by the obligate intracellular parasite *Toxoplasma gondii* is a significant cause of morbidity and mortality in immuno-compromised and congenitally-infected individuals. In livestock, abortion of ewes also causes considerable economic losses. Thus, the development of an effective vaccine against *T. gondii* would be of great value to both human and veterinary medicine. The aim of this project was to investigate whether the recombinant protein vaccines, based on SAG1, SAG2 and GRA1 *T. gondii* antigens when administered covalently attached to non-toxic murumyl dipeptide (MDP) were able to elicit humoral and cellular immune responses against *T. gondii,* in sheep.

Materials and Methods: The 25 sheep divided into five 5 equal groups were vaccinated twice by intramuscular injection with either the surface antigen (rSAG1, rSAG2) or excretory/secretory antigen (rGRA1) each individually conjugated to non-toxic (MDP). Blood samples were taken weekly. Immunoglobulins IgG1 and IgG2 were measured using an ELISA test, IFN-γ level were measured using a commercial bovine IFN-gamma test and CD4 and CD8 cells were quantified using flow cytometry.

Results: Immunisation with recombinant GRA1 protein stimulated IFN-γ production and GRA1-specific IgG2 antibody levels. In spite of the decrease in the number of $CD4^+$ and $CD8^+$ cells, immunisation with recombinant GRA1 protein is capable of inducing at least one T-cell mediated immune function (IFN-γ), which is essential for the protection against *T. gondii* infection. Levels of SAG1 or SAG2 specific IgG2 antibody in the blood serum, increased in the sheep immunised with recombinant SAG1 or SAG2 protein respectively without the production of IFN-γ *in vitro*, hence the protective efficacy of recombinant SAG1 and SAG2 protein in sheep is questionable. Immunisation with a recombinant protein cocktail vaccine enhanced the IFN-γ production of lymphocytes stimulated *in vitro* with tachyzoites, but at a level less than monovalent preparation.

Conclusion: In the present study, we have demonstrated that the intramuscular injection of recombinant vaccines based on *T. gondii* rSAG1, rSAG2 and rGRA1 proteins coupled to the non-toxic MDP, into sheep has the ability to stimulate specific humoral and cellular immune responses. Immunization with recombinant GRA1 protein gave significant cell mediated and humoral (GRA1 specific) immune responses. This preparation could be regarded as a potential vaccine candidate to protect against *T. gondii* infection in sheep. However, vaccination with SAG1, a well studied antigen, has not shown the capability of stimulating IFN-γ production *in vitro*. These results encourage further research to develop an improved vaccine against *T. gondii* in sheep.

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P15.31

Effect of selected chemical agents on bacterial laccase catalytic activity

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Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyse the one-electron oxidation of variety aromatic compounds. The use of oxygen (a non-limiting electron acceptor) by the laccases makes these enzymes adequate for industrial and environmental applications e.g.: bioethanol production, dye decolorization, detoxification of industrial effluents, bioremediation, biobleaching of paper pulp, biosensing and wine stabilization. Due to the numerous biotechnological applications of laccase, it is essential to determine the influence of different agents on its enzymatic action, as well as the effect of various ions significant for protein purification procedures.
The main goal of this work was to contribute to a better knowledge of these enzymes with respect to potential biotechnological application of laccases. In the present work, the effect of different compounds on laccase activity was determined.
Ions (Cu^{2+}, Fe^{3+}, Mn^{2+}), chelators (EDTA, imidazole), reducing compounds (reduced glutathione, β-ME, DTT, TGA-Na), electron flow inhibitors (F^-, N^{3-}, CN^-), halides (NaCl, NaBr, KCl, KJ), ionic detergents (SDS) and Na_2SO_4 at appropriate concentrations were tested for their activity toward semi-purified intracellular laccase of *Sinorhizobium meliloti* in different test systems utilising 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine) as enzyme substrates in citrate-phosphate buffer. Laccase was inhibited by Mn^{2+} in more than 95%. Whereas, chelating agents (EDTA and imidazole) reduced the enzyme activity about 45 % at a concentration of 1–100 mM. The use of halides and sodium sulfate resulted in inhibition of enzyme activity ranged from 81.5% (KJ) to 55.57% (Na_2SO_4). Laccase activity was increased by the addition of 0.1–10 mM SDS and strongly inhibited by sodium azide, fluoride (electron flow inhibitor) and dithiothreitol.

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P15.32

Molecular and functional studies of AstA sulfate transporter, unraveled protein in pathogenic fungi

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AstA protein (alternative sulfate transporter) represents a little known type of sulfate transporter, belonging to an extensive but poorly characterized family of allantoate permeases Dal5. In *Aspergillus nidulans* the *astA* gene is under the control of Sulfur Metabolite Repression (SMR) [1]. The closest homologs of *astA* are frequent in evolutionarily distant fungi belonging to the *Pezizomycotina* subphylum which exhibit similar plant pathogenicity. They are mostly crop pathogens represented by species sequenced for now: *Fusarium graminearum* (*Giberella zeae*), *F. verticillioides* (*Giberella moniliformis*), *F. oxysporum*, *Nectria haematococca* (*Fusarium solani*), *Verticillium alfalfae*, *V. dahliae, Septoria musiva, Leptosphaeria maculans*.
Molecular analysis of *A. nidulans* AstA led to identification of amino acid residues crucial for sulfate binding and transportation. Dependent of culture conditions, AstA is internalized between membrane or mitochondrion. In the fungal pathogen *Fusarium sambucinum* a high expression level of the orthologous gene *astA* was observed during infection of the potato tubers.
Due to the similarity on the metabolic level between pathogenic fungi and the host, there is a problem with efficient plant protection. Elucidation of the biological function of AstA should help understanding of fungal pathogenic adaptations upon changes in plant host metabolism and definition of a new promising target for a potential fungicide.

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P15.33

Structural studies on DNA cleavage-and-ligation nucleases of mobile genetic elements involved in spread of antibiotic resistance

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Whatever the route used, horizontal gene transfer (HGT) requires sophisticated multi-protein machinery to enable the long and charged DNA polymer to cross the cell envelope barriers. One of the best studied system for the cell-to-cell DNA translocation is bacterial conjugation, a major mechanism of genetic exchange in bacteria. Plasmids and integrative and conjugative elements (ICEs) are the major mobile genetic elements (MGEs) that provide routes for the rapid acquisition of new genetic information and contribute to the spread of antibiotics resistance. Essential for their action are plasmid/ICE-encoded structure- and sequence-specific endonucleases, historically termed relaxases (from the relaxation of the plasmid supercoiled form upon nicking). Relaxases form intermediate covalent adducts with their cognate substrate DNA and such complexes called relaxosomes are transferred to the recipient cells. Additionally, plasmids encode for plasmid DNA replication relaxases, crucial for their maintenance. Understanding plasmid conjugal transfer and replication may aid in combating plasmid/ICE-related adverse effects on human health (e.g. antibiotics resistance) as well as cotribute to the development of new tools for DNA delivery into human cells. Our interests include replicative relaxase RepB and conjugative relaxases MobM and TrwC. Structures of relaxase domains of these proteins have been solved in our lab and herein compared.

P15.34

Expression, isolation and purification of recombinant human DMP1 and its functional fragments

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Biomineralization refers to wide range of processes by which living organisms form mineral crystals. Usually those crystals are formed and deposited within the organic matrices and vesicles for different purposes. Biomineralization process is complex and not fully understood. Many proteins characterized to be engaged in biomineralization manifest character of intrinsically disordered proteins (IDPs) [1].Otoliths in bony fishes and otoconia in mammals are composite crystals consisting of calcium carbonate. These biominerals are part of the gravity and linear acceleration detection system of the inner ear. They are involved in the perception in balance, as well as in the reception of sound. Dentin matrix protein 1 (DMP1) is a noncollagenous protein of extracellular matrix predominately expressed in bone and dentin. DMP1 plays an important role in proper phosphate homeostasis and mineralization. The disordered character of DMP1 has been shown using several methods. It has been demonstrated that DMP1 protein is proteolytically processed into fragments, including 37K fragment from the NH2-terminal region and 57K fragment from the COOH-terminal region. Recently, circling behavior developed in DMP1 null mice was described and suspected to be cause due to bone defects in the vestibular apparatus. Based on the above information we decided to express DMP1 protein in bacterial expression system as well as its functional fragments 37K and 57K and purify them for further biomineralization of calcium carbonate within in vitro nucleation test. This enables the further assessment of molecular basis of biomineralization process and the role of DMP1. Constructs containing cDNA encoding DMP1, 37K and 57K proteins with His-Tag were obtained and were used to transform *E.coli* cells. Immobilized metal anion affinity chromatography (IMAC) was chosen as a first step of purification procedure. Because of high content of acidic amino acids, ion exchange chromatography with a Mono Q column was applied next.

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P15.35

Intrinsically disordered otolith matrix macromolecule — 64 controls mineralization of calcium carbonate

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Calcium carbonate and calcium phosphate biominerals are major constituents of the hard tissues or organs like bones, shells, teeth and otoliths of living organisms. They form composite crystals of inorganic materials and organic matrix. Although organic matrix is considered to control size, shape, direction of growth and polymorph of crystals still little is known about exact chemical composition, structure and function of its individual components, i.e., proteins, polysaccharides, polyamines and lipids.

Otolith matrix macromolecule — 64 (OMM-64) was found in the inner ear of *rainbow trout*. It is present in the otolith — a mineral structure, which is a part of gravity and linear acceleration detection system. OMM-64 is a component of high molecular weight aggregates (HMWAs), which control mineralization of calcium carbonate *in vitro*. Separation and identification of HMWAs components was however very difficult and little is known about structure and function of individual HMWAs components till now.

OMM-64, like many other proteins involved in biomineralization, exhibits properties of intrinsically disordered protein (IDP). The aim of this work was to investigate the effect of recombinant OMM-64 on calcium carbonate mineralization. For this purpose *in vitro* crystallization system was developed. SEM images analysis revealed that OMM-64 controls the size and morphology of calcium carbonate. Crystals formed in the absence of the organic compound or in the presence of trypsin (negative control) have cubic morphology, whereas crystals formed in the presence of OMM-64 have spherical, slightly elongated shape with stairlike structure and characteristic flat surfaces. Increase of the amount of crystals formed in the presence of OMM-64 in comparison with the control implies that the protein promotes nucleation. Size distribution of crystals grown in the presence of OMM-64 is narrow, and the crystals are smaller than control crystals. We observed increase of the crystals dimension with increasing protein concentration. The morphology and size of crystals formed in the presence of OMM-64 depends strongly on calcium ions concentration.

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P15.36

Cationic polyprenyl derivatives as efficient, non-toxic nucleic acids vehicles

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Introduction: The progress in understanding pathogenesis of diseases resulted in nucleic acids becoming actually drugs and their delivery one of the top molecular biology techniques applicable in clinics. Still, the major challenge of gene therapy is lack of efficient and safe vehicles. We examined a new class of lipids for gene transfer — polyprenyltrimethylammonium iodides (PTAI) that effectively transfect DNA into cells *in vitro* in the presence of serum, thus becoming promising candidates for *in vivo* delivery.

Aim: To evaluate features of PTAI important for successful *in vivo* application as nucleic acids vehicles. A combination of tests was exploited to examine their side effects on cell physiology, antimicrobial properties and hemolytic activity.

Methods: Cell lines: DU145 — human prostate cancer, XC — rat sarcoma, B16F10 — mouse melanoma. PTAI: synthesized from alcohols extracted from the plant tissues. Transfection efficiency evaluation: EGFP-expression assay. Viability assay: EtBr and FDA staining. Proliferation assay: manual counting in Bürker chamber. Motility assay: time-lapse monitoring of movement of individual cells. Gap junctional intercellular coupling (GJIC) test: donor cells labelled with calcein. Bactericidal activity against *S. aureus* and *E. coli*: colony reduction assay. Hemolytic activity: human red blood cells (RBCs).

Results: The results show that lipoplexes (complexes of lipids with nucleic acids) based on PTAI have no side effects on model DU145 cells physiology that is cell viability, proliferation, morphology, GJIC and motility. Some PTAI-based vectors exhibit potent bactericidal activity against *S. aureus* and *E. coli*, while showing no toxic effect on eukaryotic cells. Furthermore, we have also proved their safety towards human RBSs, which membranes are not disrupted in the presence of all the examined concentrations of PTAI-based lipoplexes. The formulations are also effective in gene silencing techniques utilizing RNA delivery (effective introduction of shRNA inducing EGFP expression silencing into DU145, XC and B16F10 cells).

Conclusion: PTAI provide ability to introduce DNA or RNA into cells with satisfying efficiency without side effects on cell physiology and show advantages important for *in vivo* applications.

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P15.37

Modified nucleic acids and their potential application in modern molecular techniques

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Synthetic nucleic acids are commonly used in scientific fields as biotechnology, biology, proteomics, medical diagnostic and gene teraphy. The polymerase chain reactions (PCR) and its modyfications (RT-PCR, real time PCR, QF-PCR and other) are increasingly used in areas that have not used the techniques of molecular biology so far. Accordingly, it is very important to develop new modified nucleic acids that are characterized by a unique propertis. Fluorogenic probes labeld by new tags make opportunities to discover subsequent ways in research.
High demand for nucleic acids, like RNA and DNA oligonucleotides, molecular probes, mixed RNA/DNA oligonucleotides, primers for PCR reaction and fluorescent-labeled oligonucleotides with the ability to fluorescence quenching, determines the research on increasing the scale synthesis of nucleic acids and the development of innovative, highly efficient supports to chemical synthesis of nucleic acids. Supports are characterized by highly developed specific surface area that enables oligonucleotides to grow in a high density structure of functional groups. Better redistribution of reagents, but not their bigger amount, will increase total yield. New supports will be based on linkers that allow to apply mild conditions of product removal from the solid phase which will enhance purity of nucleic acids. The developed highly efficient technology of synthesis in scales over 10 μmol will enable to meet the growing market demand for synthetic oligonucleotides.
Synthetic nucleic acids, particularly modyfied nucleic acids, require the selection of appropriate purification method. The optimization methods as high-performance liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE) and molecular filtration may be crucial for obtaining a high-quality nucleic acids.

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P15.38

Glucose dehydrogenase from *Aspergillus niger* — selection of the best enzyme producer and optimization of culture conditions

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The economic significance of many species of *Aspergillus* caused that these fungi became a frequent target of a scientific research. The wide range of enzymes produced by *Aspergillus* are of major importance to many diverse industries. GDH are defined as oxidoreductases that are able to catalyze the oxidation of glucose in the presence of an electron acceptor. This enzymes have been classified into three distinct groups according to their electron acceptor including PQQ, FAD and NAD-dependent enzymes Nowadays we observed the increasing use of GDH-FAD in bioelectrochemistry. GDH due to its electrochemical activity is employed as a glucose sensor or a biofuel component [1-3]. The aim of this research was to selection of the best GDH producer among 13 different *Aspergillus* strains with overproduction of glucose dehydrogenase and optimization of culture condition for this enzyme. *Aspergillus niger* (13/48) was choosen in a screening investigation as one of the best overproducer of glucose dehydrogenase. Studied strain was identified using ITS region sequencing (accession number-KF154408). The basic culture parameters like carbon and nitrogen sources, temperature and pH influencing enzyme synthesis in shaken cultures (24 well plates) were evaluated to improve the yield of the process. The optimal combination of the media constituents for GDH production was as follows: sacharose 50 g/l, urea 3 g/l, $MgSO_4 \cdot 7H_2O$ 0.15 g/l, KH_2PO_4 0.18 g/l, $CaCO_3$ 35g/l and 5 ml/l Tween 80. Maximum GDH activity were observed at 30°C in the medium of pH 5.

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P15.39

A novel platform for identification of antimicrobial cyclic peptides

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Resistance of bacteria to currently known antibiotics is increasing. It is combined with drying of discovery pipelines of pharmaceutical companies. As a result, infectious diseases constitute again a serious threat for public health. There are numerous classes of antimicrobial agents that act on different targets. One of them are membrane disturbing antimicrobial peptides (AMPs). Due to the general mechanism of their action bacteria rarely develop resistance to them. On the other hand, because of relatively low specificity and high toxicity, membrane disturbing AMPs found little application as drugs up to now. Therefore, there is a need to find new AMPs and to improve the existing ones. There are few approaches to screen for peptides with antibacterial properties. One of them is screening of chemically synthesized short random peptide libraries for bacterial growth inhibition. Such approach, however, is expensive and time consuming. Here we present a new approach to select antibacterial peptides. As a result we were able to identify toxic CPs that inhibit bacterial growth. Sequencing revealed that toxic CPs posses from one to three arginines often accompanied by tryptophan — the feature recognized previously in many of membrane disturbing AMPs. Our results provide a proof for applicability of a novel platform for identification of new AMPs.

P15.40

An effective method for cryoconservation of chicken primordial germ cells based on flow cytometry analysis

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Cryoconservation of primordial germ cells (PGCs) may be a simple way for preserving biodiversity of chicken breeds endangered with extinction. Genetically modified PGCs can be also used to production of transgenic chicken. The material for research were chicken PGCs isolated from bloodstream of 3-day old embryos (bPGCs) and from gonads of 6-day old embryos (gPGCs) and purificated by Percoll density centrifugation. The aim of our study was to compare the influence of different origins of PGCs, four cryoprotectants and four slow freezing programs on the PGCs viability and apoptosis measured via microscope after trypan blue staining and flow cytometry analysis after using Annexin V kit. Statistically significant (p0.05) differences between viability of PGCs based on trypan blue exclusion or Annexin V test were observed. Application of DMSO for slow freezing of PGCs allowed to obtain statistically significant (p0.05) higher viability of PGCs after thawing than MIX. In this study the effective method for slow freezing of PGCs based on application of 10% DMSO F and cooling rate of 2°C/min. was elaborated. Thismethod allowed to obtain the high percentage of living PGCs (93.1) and lower percentage of PGCs in early stage of apoptosis (6.5). This method was chosen for the *in vivo* test. The cryoconserved/freshly isolated PGCs were transfected with pEGFP-N1 plasmid, cultured with selective antibiotic and injected into recipent embryos. The EGFP gene was detected in gonads of 40% and 39% recipient embryos injected with cryoconserved and modified bPGCs/ gPGCs and 45% and 44% of recipient embryos injected with freshly isolated and modified bPGCs/ gPGCs, respectively. These data showed that elaborated effective system for cryoconservation of PGCs may be a useful tool for ex situ strategy of poultry biodiversity preservation.

P15.41

Overcomming drug resistance by new polymeric–phospholipidic hybrid micelle delivery system

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Appearance of drug resistance is widely spread in pathogenic microorganisms and tumor cells under chemotherapy. Nanoscale preparations offer the opportunity to overcome these drawbacks reflected in the recent approval of two liposomal Dox preparations. However, most of these strategies increased drug uptake solely in the background of acquired Dox resistance. Here, we tested a new PEGylated polymeric–phospholipidic hybrid micelle delivery system which distinctly enhanced the accumulation and activity of Dox in all cancer cell models tested.
Notably, anticancer activities were increased up to about 20-fold independent of previous Dox selection and resistance mechanisms. The nanoparticle formulation distinctly enhanced the intracellular accumulation of Dox. In SW1573 cells, this resulted in about 10-fold higher Dox levels upon treatment with PC-Dox as compared to cells treated with free Dox. Also in the Pgp-overexpressing subline 2R160 a distinctly increased intracellular accumulation of the nanoparticle-bound Dox (6-fold) was observed. It is well known that the induction of oxidative DNA damage plays a major role in the anticancer activity of Dox. Indeed, after 3 h treatment PC-Dox was distinctly more effective in enhancing the tail moment of treated cells than free Dox. In accordance with this data, treatment with the nanoparticle formulation led to stronger stimulation of stress pathways indicated by upregulation of P53 and phosphorylation of the mitogen-activated protein kinase P38. This was accompanied by a distinct G2/M arrest of the surviving PC-treated cells after 24 h treatment. Moreover, the new hybrid micelles were shown to rapidly (within 10 min) and effectively transport the drug into Dox-selected and -unselected cancer cells via endocytosis. *In vivo*, the nanoformulation already at low concentration (0.1 mg kg^{-1}) cured all treated mice from two cancer xenograft models, while free Dox solely extended survival time.
This indicates that the incorporation of phospholipids into the structure of PEGylated polymer micelles is a promising strategy to enhance efficacy and reduce toxicity of Dox *in vitro* and *in vivo* independent of the involved resistance mechanisms.

P15.42

Stability studies on hydrophobic functionalized nanoparticles incorporated into micelles

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Noble metal nanoparticles (NPs) are considered as perspective material for anticancer and antibacterial applications. It has been proved that nanometric silver nanoparticles are able to penetrate into the selected tissues and bacteria cells, leading to their apoptosis. The efficiency incorporation into the cancer and bacteria cells is higher than into unchanged tissues. This can be used to destroy cancer cells as well as for drug encapsulation and target development into selected cells.
In this contribution, we present the results of the investigation of hydrophobic functionalized silver nanoparticles introduced into different types of aqueous surfactant solutions with different ionic properties (negatively charged sodium dodecyl sulphite, SDS, neutral Triton X-100 and positively charged tetredodecyltrimethyl ammonium bromide, TTABr). The suspension of Ag colloidal nanoparticles in hexane were injected rapidly into the solutions with concentrations below and above the reported critical micelle concentration, CMC, of surfactant. Obtained system were investigated using absorbance spectroscopy and dynamic light scattering, DLS, methods.
The efficiency of nanoparticles incorporation into the micelles is highest for Triton X-100, lower for negatively charged SDS whereas for cationic micelle no incorporation occurs. In presence of SDS the aggregation of nanosilver takes place prior to transition into micelle thus the large aggregates of nanoparticles are incorporated into micelles. In case of Triton X-100 each nanoparticle is incorporated separately into vesicle. In presence of cationic detergent the silver nanoparticles form aggregates and do not interact with TTABr micelles. Obtained results indicate that in aqueous environment the properties of surfactant molecules and concentration ratios between components determine the efficiency and kinetics of nanoparticles between two competitive association or aggregation processes.
Reported preliminary results indicate direction for further investigations leading to determine the optimal conditions for incorporation of Ag NPs into surfactant solutions to produce stable and reproducible Ag/surfactant systems for antibacterial applications.

P15.43

Cytotoxic conjugates based on the *affibody* molecule that target HER2-positive cancer cells

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Development of efficient and safe cancer therapy is one of the major challenges for modern medicine. Targeted therapy is a newer type of cancer treatment that employs biologically active drugs attached to monoclonal antibodies or other ligands *via* chemical linkers with labile bonds. This approach allows to use highly toxic substances that specifically target tumor cells therefore healthy tissues are less affected than in the conventional chemotherapy.

Overexpression of HER2 (Human Epidermal Growth Factor Receptor 2) occurs in about 20%–30% of invasive breast cancers and correlates with poor prognosis [1]. Commercially available monoclonal antibody against HER2 receptor known as Herceptin has been used in the treatment of HER2-positive cancers. However, therapies based on Herceptin turned out to be inefficient. Kadcyla (Genentech), an antibody-drug conjugate containing Herceptin linked to the cytotoxic agent mertansine (DM1) has been approved for treating HER2-positive breast cancers in 2013 [2]. This strategy is supposed to be far more successful than the previous Herceptin-based therapies.

The aim of our work is to develop and characterize a new cytotoxic conjugate against HER2-positive tumors. The conjugate contains auristain E, a highly potent drug that blocks tubulin polymerization and inhibits cell division. In our research we use a small protein, affibody $Z_{HER2:2891}$ that recognizes HER2 receptor with high affinity, as a targeting molecule [3]. Affibody $Z_{HER2:2891}$, does not contain cysteines in its amino acid sequence. Therefore, a single cysteine was introduced into different sites in the amino acid sequence of affibody $Z_{HER2:2891}$ to allow conjugation between the cysteine thiol group and the maleimide group, that is present in the cytotoxic component. Currently, obtained conjugates are being tested in the cytotoxicity assays.

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P15.44

Taq DNA polymerases fused with single-stranded DNA binding protein

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DNA polymerase is an enzyme which plays crucial role in replication and DNA repair. It found application in PCR (Polymerase Chain Reaction) where catalyses process of *in vitro* DNA synthesis by adding nucleotides to 3'OH end of DNA strand. DNA polymerases have broad range of application in molecular biology, genetic engineering and molecular diagnostic [1]. One of the most important steps in polymerisation activity of these enzymes, which is responsible for their final efficiency, is initiation step connected with binding to matrix DNA. Therefore, it is reasonable to modify well known DNA polymerases in order to facilitate binding to polymerised DNA strand.

Example of such modification may be creation of fusion DNA polymerases with proteins which naturally bind to single and double stranded DNA. However there is only a few examples described in scientific literature. Research shows that fusion of *Taq, Pfu* and *Tpa* DNA polymerases with protein binding double or single stranded DNA resulted in increasing of processivity from 5 to 17 fold [2-5]. There is lack of information about fusion of the most frequently used in molecular diagnostic *Taq*polymerase with single stranded binding proteins. For this polymerase also scientific papers describing fusions with a peptide linker are not available.

The aim of our study was determined the influence on particular properties of *Taq* DNA polymerase essential in molecular diagnostic and genetic engineering.The polymerase was connected with DNA binding proteins by 6-amino acids linker (Gly-Ser-Gly-Gly-Val-Asp). Linker will provide the fusion protein more flexibility and relaxed arrangement to polymerase and will allow avoiding a steric hindrance what may be crucial in the process of binding to DNA and polymerisation. In the fusion was used protein binding single stranded DNA (RB69SSB). This is small protein which is active as a monomer. It isisolated from thermophilic microorganisms what suggests its thermostability what is essential in PCR where temperature of denaturation step exceeds 90°C.

The present studies suggest that this fusion resulted in increasing of reaction efficiency and processivity.

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P15.45

Multi-well *E. coli*-based platform for optimal production of recombinant proteins

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The novel system of ligation independent cloning for generation of genetic constructs designed for the production of proteins of interest (POIs) in *E. coli* has been applied. The system is based on the modular approach, in which the indispensable elements of the expression vector such as the promoter, selection marker, origin of replication, and gene of interest are generated by the PCR. Each module contains the complementary fragments that allow joining the modules in the specific PCR reaction, termed the circular polymerase extension cloning. The modules occur in versions, which allow the generation and selection of the genetic constructs with the highest levels of production of POIs. The clones are cultivated under the expression inducing conditions and the levels of production of POIs are assessed in the multi-well plate format. The clones with the highest gene expressions are used for scaled-up production of POIs, which are purified using the affinity chromatographies based on common fusion tags. Each genetic construct contains the Clean-Cut recognition site, which together with Clean-Cut a novel, proprietary, unique and highly specific recombinant serine protease facilitate the tag removal. The presented approach is cost and time effective and is ideal for determination of the optimal conditions for the overexpression and production of the range of recombinant and heterologously produced proteins surpassing the standard cloning and expression tests.

P15.46

Effect of copper and cadmium ions on *in vitro* degradation of a full-length protein substrate by fungal 26S proteasomes

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The majority of proteins in eukaryotic cells are degraded by a highly selective non-lysosomal pathway that requires ATP and a large multicatalytic proteinase complex known as the 26S proteasome [1]. The 26S proteasome is dedicated primarily to the degradation of proteins tagged with polyubiquitin chains. There are exceptional cases in which degradation by the 26S proteasome requires ATP hydrolysis but not ubiquitination [2]. In our previous studies we have established conditions in which the proteasomes isolated from mycelia of the white rot fungus *Trametes versicolor* (as described in [3]) degraded a full-length protein substrate (β-casein) in ATP-dependent manner in the absence of ubiquitination [4]. White-rot fungi require trace amounts of essential heavy metals for their growth, but these metals are toxic when present in excess [5]. It has been previously observed that Cu^{2+} and Cd^{2+} at low concentrations increased laccase activity in *T. versicolor* cultures [3]. Laccase is considered one of the key enzymes involved in lignin degradation by white rot fungi, well known for its biotechnological applications. In the present study, we investigated the effect of Cu^{2+} and Cd^{2+} on *in vitro* digestion of β-casein by the 26S proteasomes isolated from mycelia of *T. versicolor* using electrophoretic and spectrofluorimetric analyses [6]. The spectrofluorimetric method using the reaction of degradation products with fluorescamine to measure the rate of protein degradation by the proteasomes was shown to be a good alternative to procedures with the use of radioisotope-labeled protein substrates. We found that Cu^{2+} and Cd^{2+} reduced the degradation rate of β-casein in a dose-dependent manner. Cadmium ions at the lowest concentration used (2μM) decreased β-casein digestion to approximately 30%. The present study implies proteasome-mediated proteolysis by the 26S proteasomes in the response of ligninolytic fungi to heavy metal ions.

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P15.47

New strategy to mobilize bone marrow cells to the blood: unexpected application of cobalt protoporphyrin IX

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Cobalt protoporphyrin IX (CoPP) is a known inducer of heme oxygenase-1 (HO-1). Apart of the heme breakdown, HO-1 has many other functions — it was shown to be a proangiogenic, anti-apoptotic and immuno-modulatory factor. The aim of the study was to evaluate the effect of HO-1 induction by CoPP on myeloid cell populations.

C3H and C57BL6xFVB mice were injected intraperitoneally with CoPP at a dose of 10 mg/kg, three times, at intervals of two days. Control mice received only the solvent (DMSO). The day after the last CoPP injection, the mice were sacrificed and peripheral blood, bone marrow and spleen samples were collected.

The results of blood morphology analysis indicate that mice treated with CoPP have increased number of leukocytes in the blood compared to mice that received only DMSO (3-fold increase in C3H strain, 2-fold increase in C57BL6xFVB strain). In both strains, there was a significant increase in the number of granulocytes. There were no changes in blood parameters such as hematocrit or the number of erythrocytes.

The populations of leukocytes, which were affected by CoPP administration, were characterized in more detail using a flow cytometer. Treatment with CoPP resulted in increased number of immature granulocytes ($CD11b^{+}Ly6G^{low}Ly6C^{-}SCC^{mid}$) in the blood.

In order to investigate the mechanism responsible for the increase in the number of leukocytes, the concentrations of cytokines and growth factors in the blood plasma using the Luminex system. We observed increased levels of granulocyte colony stimulating factor (G-CSF, 96-fold increase in C3H strain, 57-fold increase in C57BL6xFVB strain), chemokines: MCP-1 (CCL2, 162-fold increase in C3H strain, 15-fold increase in C57BL6xFVB strain) and IP-10 (CXCL10) and interleukin 6 in mice treated with CoPP compared to mice treated with DMSO.

To conclude, administration of CoPP increases the amount of G-CSF in the peripheral blood, which results in the mobilization of leukocytes, mainly immature granulocytes, into the bloodstream. At the same time the production of CoPP is potentially less complicated and less expensive than the recombinant protein G-CSF analogs which are currently used for clinical applications, such as treatment of neutropenia induced by chemotherapy.

P15.48

Comparison of the properties of non-immobilized and immobilized synthetic protease inhibitors

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Proteolytic enzymes (also termed as peptidases, proteinases and proteases) play essential roles in many biological processes, especially in cell metabolism. They are involved for example in digestion process, embryogenesis, blood coagulation and immune response [1]. Despite their very important regulatory functions, enzymes that break down protein bonds are potentially very dangerous for living organisms. Therefore, natural proteolytic activities need to be strictly regulated and kept under control. There are several mechanisms for the control of protease activity but one of the most important among them is inhibition of proteolytic activity by natural or synthetic protease inhibitors [2]. This small molecules or peptides can also be versatile tools in field of many industries like biotechnology, medicine or agriculture [3].

In the present study the properties of immobilized and non-immobilized synthetic inhibitors of proteolytic enzymes were investigated. As the immobilization carrier we used controlled pore glass. Immobilization conditions were initially optimized for selected inhibitor. After the immobilization process the storage stability, the stability under different conditions of pH and temperature values or the antimicrobial properties for immobilized inhibitor were tested in comparison to its free form. Obtained results showed that the immobilized inhibitor is much more stable than non-immobilized inhibitor and may offer a wide range of biotechnological applications.

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P15.49

Aspartyl protease from psychrotolerant yeast *Sporobolomyces roseus*

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About 85% of the biosphere is psychrosphere, where the average temperature during the year does not exceed 5°C. All these areas are colonized by psychrophilic or psychrotolerant microorganisms. The ability to survive in such extreme environmental conditions can be acquired only through microbial adaptations. One of their most important adaptation to living in cold environments is the production of thermolabile enzymes that are able to efficiently catalyze reactions occurring at low temperatures. In addition, structural and kinetic properties of psychrophilic enzymes make these proteins interesting for various biotechnological applications. They are characterized by two important features - high catalytic activity at low temperatures and also the most low thermal stability, which make them attractive catalysts in industrial processes, mainly due to the economic benefits of their use, including energy savings by avoiding heating bioreactors and related energy expenditure.
One of the most common groups of cold-adapted enzymes are proteases, which are known as the most commercialized group of enzymes. Proteases have found applications in diverse fields such as detergent industry, leather processing, silk degumming or food and dairy industry. Currently we more often speaks about their use in unconventional environments for the synthesis of peptides, potentially useful for the pharmaceutical industry.
The aim of research was to isolate psychrotrophic yeast strains from environmental samples and conducting a screening of the isolates for a high extracellular proteolytic activity.
One of the most efficient producers of extracellular proteases was a psychrotrophic yeast strain genetically classified as *Sporobolomyces roseus*. Further studies showed that this strain is the producer of the aspartyl protease of relatively high activity of 1.5 U/ml. This enzyme was purified to homogeneity by using ion-exchange chromatography and its properties was analyzed. The optimum pH for enzyme activity is 4, and the optimum temperature is 50°C. This enzyme, despite of high for a cold adapted protein optimal temperature, shows a relatively high activity in the temperature range 0–20°C (respectively 10–25%). Furthermore, the enzyme is a typical thermolabile protein, which undergoes thermal inactivation after 30 minute preincubation at 40°C.
Aspartyl protease produced by *Sporobolomyces roseus* appears to be an interesting biocatalyst in terms of biochemical properties and biotechnological potential. It is also worth emphasizing that as so far described in the literature cold-adapted extracellular proteases are mainly produced by bacteria. There are only two reports of the production of such adapted to cold proteases by yeast.

P15.50

Toxicity evaluation of two generations of viologen-carbosilane-phosphorus (SMT) dendrimers against the B14 and N2a cell lines

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Almost unlimited field to explore is given by dendrimers. Constantly synthesized new classes of dendrimers offer almost endless possibilities of their usage. Often, the well-known classes of dendrimers give rise to the formation of the new type with truly surprising properties. As a class of well-known dendrimers we can consider phosphorus dendrimers (PD), carbosilane dendrimers (CBS) and viologen-phosphorus dendrimers (VPD).
Phosphorus hydrophobic core together with hydrophilic surface groups constitute the structure of phosphorus dendrimers. The extremely important for the interaction with biological systems is the presence of the phosphorus atom. Due to the specific properties P-dendrimers are candidates suitable in the treatment against HIV virus. Worth mentioning is the fact that P-dendrimers possess anti-HIV activity at micromolar concentration. However, this type of dendrimers is also highly cytotoxic, what can be an obstacle in anti-HIV therapy.
On the other hand, the presence of the silicon atom is a specific feature of carbosilane dendrimers. Kinetic and thermodynamic stability in this type of dendrimers is performed by the high energy of carbon-silicon binding. The synthesis of water soluble CBS dendrimers with various surface groups creates the prospect for medicine. It has been proven that sugar residues allow the connection of CBS dendrimers with the receptors exposed to viruses. Conclusion: CBS dendrimers are potential candidates for antiviral drugs. In turn, CBS dendrimers with carboxyl end groups can reduce the level of HIV infection, although the mighty activity against HIV is held by CBS dendrimers with sulfate and naphthylsulfonate surface groups.
Comparing to cationic dendrimers such as PAMAM and CBS, viologen-phosphorus are characterized by the low cytotoxicity. This relatively low toxicity may seem surprising considering the fact that dendrimers VPD were created by combining toxic viologen groups and exhibiting high toxicity phosphorus dendrimers. VPD owe their low toxicity to cationic groups placed within the molecule.
Cationic phosphorus and carbosilane dendrimers also have the potential to become efficient carriers of siRNA in gene therapy. So, they give hope to be effectively used as siRNA carriers in anticancer therapies. Possibilities and limitations of mentioned above dendrimers contributed to the creation of a new class of dendrimers constitute their combination. For these reasons, it is worth checking whether a new class of dendrimers (viologen-carbosilane-phosphorus – SMT) is toxic toward cells.
The aim of the study was to evaluate the toxicity of two generations of viologen-carbosilane-phosphorus dendrimers by measuring their influence on B14 and N2a line. To achieve the objective, MTT assay, flow cytometry with H_2DCFDA probe and JC-1 assay were conducted. All of

the assays were carried out on Chinese hamster fibroblast cells (B14) and mouse neuroblastoma cell line (N2a).

On the basis of the results, it was observed that dendrimers decreased cell viability, induced changes in mitochondrial potential and increased the ROS level enhancing with the concentration used. The differences between the generations of SMT dendrimers and the cell lines were noticeable.

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P15.51

Development of plant systems expressing human lactoferrin for biopharming and enhanced resistance to pathogens

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Lactoferrin (Lf), one of the promising human milk proteins, occupies the expanding biotechnological food market niche due to its multiple properties. Thus, Lf exhibits antiviral, antimicrobial, antiprotozoal and antioxidant activities, modulates cell growth rate, binds glycosaminoglycans and lipopolysaccharides, and also inputs into the innate/specific immune responses. Development of the highly efficient human recombinant Lf expression systems employing yeasts, filamentous fungi and undoubtedly higher plants as bioreactors for the large-scale Lf production is considered to be the challenging task (Yemets *et al*., 2014). Moreover, due to various protective Lf properties, its genes are desirable candidates for the introduction into the genomes of economically important higher plant species for the enhancement of their immune response, and resistance to different pathogens and diseases. Hereby, two systems, barley (*Hordeum vulgare* L.) as monocot and tomato (*Lycopersicon esculentum* Mill.) as dicot, were used for the transformation with the human Lf (*hLf*) gene. For this purpose two constructs: pBiLF carrying *hLF* gene driven under glutelin promoter and pBinar35LF driven *hLf* under 35S promoter were created (Tanasienko *et al*., 2011). Firstly, we elaborated systems for the transfer and successful expression of recombinant *hLf* in mature embryos and callus cultures of several commercial barley varieties via *Agrobacterium*–mediated and biolistic transformation. In order to elaborate the successful transformation protocol, the regeneration potential of eleven commercial cultivars of spring barley was studied. Taking into account a high level of somatic embryogenic capacity of four varieties, they were used for further genetic engineering manipulations. Also, a range of tomato explants (hypocotyls, cotyledons and apical meristem) were investigated for the same purpose. A number of transgenic barley and tomato plants were selected successfully after *Agrobacterium*-mediated/biolistic transformation. The transgenic nature of obtained plants was confirmed by molecular genetic analysis. Thus, human Lf expression levels in different lines were tested by the extraction of total RNA from 2 days-old germinated seeds using TRizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Subsequently, the total RNA preps were additionally purified by RNeasy KIT (Qiagen, Valencia, CA). 5 μg RNA was used to synthesize first strand cDNA with the Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR was applied to 15 ng of cDNA with lactoferrin specific primers and carried out using Taq polymerase (Fermentas, Vilnius, Lithuania). The 542 length fragment of the *hLf* gene was amplified during molecular analysis from transgenic lines that confirm a successful integration and expression of recombinant *hLf* gene in the transgenic plants. The study of pathogen resistance/tolerance in produced lines is under progress.

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P15.52

Quality assessment of modified and unmodified siRNAs for molecular biology applications

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RNA interference is one of the most important discoveries in molecular biology over the last decade. It has become a very significant and useful tool in cancer diagnostics and treatment [1], viral [2] as well as in neurodegenerative diseases therapy [3]. Delivering into the cell non-native RNA with intended strong specificity demands a high quality sample. Otherwise, the process translates into unspecified regulation of gene expression or its lack. Hence, marking its quality is often essential for RNAi efficiency. Chemical modifications of RNA are used to bring more stability into molecules. These modifications are aimed at improving RNA resistance to nucleases activity, boosting interference process, increasing the melting temperature of siRNA duplexes, regardless of whether they are introduced into the base, sugar or backbone [4].
In this study PVA-coated capillary gel electrophoresis was used as a technique recommended for purity assessment of siRNA, since the presence of shorter sequences limits the use of siRNAs. This finding is based on comparative studies between reciprocally competitive techniques: HPLC and CGE. The HPLC analysis briefly informs of the presence of shorter RNA. Owing to the low level of separation it is difficult to estimate the number and length of shorter oligoribonucleotides. The analysis of the same sample looks different using the PVA-CGE mode. It allows one to separate the mixture more precisely and ascribe initially individual signals to the oligomers of a concrete length, including shorter sequences. Extensive scientific research has proved the 5'-phosphorylation form of the siRNA to be an important determinant of generating an RNA-induced silencing complex (RISC) [5]. Chemical synthesis of nucleic acids enables the synthesis of such oligomers with and without a phosphate moiety on 5' or 3' end. In this context, determination of purity of modified oligoribonucleotides and discerning the phosphorylated and non-phosphorylated forms using CGE is also presented.

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P15.53

BMV- and RCNMV-based virus like particles as carriers of biologically active substances

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One of the challenges of current biotechnology is to improve the existing and to develop new diagnostic and therapeutic methods. Novel highly advanced materials and nanoparticles open a number of new opportunities in this field. One of them is an application of nanoparticles as drug carriers. So far several types of nanoparticles have been developed. Among them liposomes, dendrimers, quantum dots (QD), polymer vesicles and virus like particles (VLP) are the most widely used and tested. Each of them has some advantages; however, carriers based on viruses seem to be especially promising. They can be produced in large amounts in relatively short time, they are biodegradable and biocompatible. VLPs can be made from bacteriophages, animal or plant viruses. Huge advantages of plant viruses are that they are not pathogenic for humans, they have self-assemble properties and they production in plant hosts is relatively cheap and fast. The structures of capsids of many plant viruses have been also well known what is necessary for further research. However, the effective plant virus-based carrier is still missing.
In research herein we have examined two potential carriers: brome mosaic virus (BMV) and red clover necrotic mosaic virus (RCNMV). We have tested two systems of VLP production. The first one uses plant hosts and results in large amount of viruses that can be easily used as a carrier. The second one is based on capsid protein production in a bacterial system and virus assembly *in vitro*. The latter enables to modify capsid protein structure and in this way to change the characteristics of the carrier. To form a functional VLP with an active factor inside we have also examined two methods. The first one relies on the application of different pH conditions which induce the changes in the capsid structure. This method enables the active factor to migrate inside the capsid. In the second a dialysis-based method, recombined capsid proteins are used to encapsidate active substance *in vitro*.

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P15.54

Optimization of growing medium composition for laccase- and peroxidase-producing fungal biomass in terms of textile dyes synthesis

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White rot fungal Basidiomycota, well-known producers of laccase, are also able to secret different peroxidases, and these listed enzymes are involved in lignin modification and degradation. Low substrate specificity of laccase makes this enzyme one of the most popular biocatalyst for industrial purposes, including dyes synthesis for textile industry. However, a production of laccase on industrial scale still is very expensive and because of this the use of laccase-producing fungal biomass is alternative solution available for industrial applications such as textile dyes synthesis. As some of laccase-producing fungal biomass can secret as well as laccase also significant amount of peroxidase, which may oxidize dyes precursors, therefore the best strain for dyes synthesis is the one secreting high laccase and low peroxidase amount.

In this study, a capability of fungal biomass for laccase and peroxidase production was tested. The process was conducted with using four different growing media during the growth of four Basidiomycota fungi: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Nematoloma frowardii,* and *Rhizoctonia praticola*. During the growth of all tested fungal strains, the activities of laccase and peroxidase were measured. Furthermore, immobilized biomass of all strains was used in transformation of selected precursors to colourful products during the growth on selected medium. The results were compared in terms of laccase and peroxidase activities and time of the highest activities was specific for each strain and depended on the composition of growing medium. The intensity of coloured product synthesis by each strain and time of this coloured product biosynthesis were also specific for each strain.

Laccase can be replaced by fungal biomass acting as a whole-cell biocatalyst with properties comparable to the isolated form of the enzyme. The application of the whole-cell system simplifies the transformation process and reduces the time required for its completion.

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P15.55

Histamine augments glycosaminoglycans content in cultured fibroblasts isolated from the granulation tissue of skin wound

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Histamine was proved to accelerate cutaneous wound healing, and to increase collagen and glycosaminoglycans accumulation in the granulation tissue. However, mechanism by which histamine affects wound healing remains unknown. The present study was aimed at explanation whether histamine acting directly on fibroblasts isolated from wound granulation tissue plays a role in glycosaminoglycans accumulation. Moreover, we identified histamine receptors possibly involved in this process.

To this end, polypropylene net was implanted subcutaneously in male Wistar rats. After a 4-week period, fibroblasts were isolated from the implants covered with the granulation tissue. The cells were incubated with histamine at concentrations of 10^{-6}–10^{-8} M. Then, using histamine receptor antagonists ketotifen, ranitidine, ciproxifan, and JNJ7777120, known to inhibit the activity of respectively, histamine receptor H1, H2, H3, and H4, the effects of histamine receptor blockade on the collagen/glycosaminoglycans content in the fibroblast cell culture was investigated. Histamine increased glycosaminoglycans level at all concentrations used and the blockage of the H3 receptor by ciproxifan reduced this effect. The other inhibitors of histamine receptors were not effective. The present study showed that histamine increased deposition of glycosaminoglycans, in the cultures of fibroblasts isolated from the granulation tissue of skin wounds and this effect was mediated by the H3 receptor.

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P15.56

Caffeine modulates mutagenic activity of a food-borne carcinogen AαC by noncovalent complexes formation

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Diet is one of the most important factors contributing to the development of cancer. Increasing number of cancer incidents, especially in Western diet countries, is often attributed to heterocyclic aromatic amines (HCAs). In contrast to the majority of HCAs, 2-amino-9*H*-pyrido[2,3-*b*] indole (AαC) is formed in a creatine-independent process, therefore it is present in foods of both plant and animal origin. Currently it is believed that AαC is one of the most abundant HCAs in a human diet, present not only in meat and fish, but also in vegetables and mushrooms. On the other hand, diet is a rich source of many biologically active compounds with anticarcinogenic properties. Numerous epidemiological studies revealed that caffeine (CAF), the most frequently consumed alkaloid worldwide, can reduce the risk of several cancer types. It is hypothesized that CAF can act as an interceptor, sequestering aromatic mutagens in noncovalent molecular complexes, and in consequence decreasing their bioavailability. The aim of the study was to assess whether CAF forms noncovalent complexes with AαC and to investigate the possible role of such interactions in modulation of AαC mutagenic activity.

We employed UV-Vis absorption spectroscopy measurements in order to examine direct interactions between AαC and CAF. Observed spectral changes indicated mixed complexes formation between analyzed compounds. The statistical-thermodynamical model was used to calculate concentrations of all mixture components and to determine neighbourhood association constant values (K_{AC}). This analysis also showed that for the highest CAF concentrations (CAF : carcinogen ratio about 90 : 1), only less than 20% of AαC molecules were present in a free form. Next, we applied bacterial mutagenicity assay with *Salmonella typhimurium* TA98 strain to investigate CAF impact on AαC biological activity. We observed that CAF caused a dose-dependent, statistically-significant decrease in AαC mutagenic activity. Moreover, we demonstrated that mutagenic activity of AαC and CAF mixtures were strongly associated with the presence of a mutagen in a free, biologically available form.

The findings of this work suggest that CAF interacts directly with food-borne mutagen, belonging to heterocyclic aromatic amines AαC and inhibits mutagenic activity of this mutagen. These observations strongly suggest a key-role of interceptor CAF properties in its protective effects against food-derived aromatic carcinogens.

P15.57

The factors determining the successful and effective *in vitro* refolding of human adenylate kinase

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The expression of heterologous proteins in bacterial hosts frequently leads to their aggregation and deposition in dense, insoluble and mostly inactive particles, so-called inclusion bodies. Still the production of recombinant proteins as inclusion bodies is favored for several reasons. The inclusion bodies can be easily isolated due to density differences and may consist almost exclusively of recombinant proteins. Moreover, the storage of recombinant proteins as aggregates features distinct protection from protease degradation and the expression in inclusion bodies protects the cell against the toxicity of the heterologous proteins. However, there is a lack of universal and effective method for recovery of biologically active and soluble protein in high yield. The *in vitro* refolding procedure poses a bottleneck in every downstream scheme and it should be precisely adapted to the requirements and features of the target protein.

Our studies aimed at identification of chemical as well as physical environment ensuring the *in vitro* reactivation of recombinant human adenylate kinase isoenzyme 2. This mitochondrial phosphotransferase catalyzes the interconversion of adenine nucleotides and participates in cellular energy homeostasis. The *in vitro* refolding approach based on simple method of dilution combined with further dialysis. With the purpose of identification of the most effective and accurate conditions for proper folding of the enzyme a several variables were investigated. The final protein concentration, time and temperature as the main operation parameters of *in vitro* refolding process were analyzed. On the assumption that the refolding buffer is one of the crucial parameters affecting the final success, we used a several refolding buffers of differing pH, ionic strenght and presence of additives known as factors which may either promote folding of the protein or inhibit aggregation. Our results clearly indicate that the high ionic strenght as well as the presence of a reducing agent and an amphiphile in a refolding buffer provide necessary conditions which allow proper folding and formation of the enzymatically active adenylate kinase.

P15.58

Promoter of the ribulose-1,5-bisphosphate carboxylase small subunit gene in C4 plant biotechnology

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Genetic modification of the plant genome is a well-established technology applied to study genes function as well as to improve plants agronomical properties. Specific promoters addressing the expression of the gene of interest to a proper tissue and organ are crucial tools in a plant genetic engineering. Dicotyledonous and monocotyledonous plant promoters of ribulose-1,5-bisphosphate carboxylase small subunit (rbc ss, rubisco small subunit) genes were applied in a variety of constructs for plant nuclear genome modification. Depending on the type of plant photosynthesis, the rbc ss promoter addresses expression either to leaf mesophyll cells - in plants of C3 type photosynthesis or to bundle sheath cells – in C4 plants.
We conduct research into rbc ss promoter form *Sorghum bicolor* - C4 plant of high agronomical importance increasingly used as "energy crop". In contrast *to Oryza sativa*, *Triticum aestivum* or *Zea mays*, there is only a single copy of Rbc ss gene - Sb05g003480 in the sorghum genome. A phylogeny tree of rbc ss peptides reveals that Sb05g003480 peptide is the most closely related to *Z. mays* peptide NP 001105294. Also promoters of these two genes reveal essential structural similarities. The motifs involved in light dependent regulation, I-box, G-box and CG rich elements, are located proximally to TATA box and transcription start site. The architecture of cis-regulatory units (promoter region) of sorghum rbc ss gene suggests that it is highly expressed, light-responsive and photosynthesis associated [Weeks-2007, Lopez-Ochoa-2007]. The expression of Sb rbc ss gene analyzed with RT-PCR and investigated by ESTs database screening suggests its association with photosynthesis.
The goal of this study is to characterize the promoter of Sb rbc ss gene, to identify regulatory motifs responsible for its expression in bundle sheath cells and to convert the promoter of this gene into a convenient tool for C4 monocotyledonous plants genetic engineering.

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P15.59

Alpha-ketoglutarate inhibits proliferation, migration and mineralization of human osteosarcoma (Saos-2) cells

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Introduction: Osteosarcoma (OS) is a primary bone tumor, affecting mainly adolescents, characterized by aggressive clinical course with bone and soft tissue destruction and the tendency to early metastases (predominantly to lungs) [1].
Alpha-ketoglutarate (AKG) is a key intermediate in Krebs cycle. It is involved in the formation of amino acids, nitrogen transport, oxidation reactions and it can also function as a regulating and signaling molecule. AKG is present in the human cells and it is already commercially available as a dietary supplement. Antitumor activity of AKG has been demonstrated in several studies. It is based on the inhibition of tumor growth in hypoxic conditions (reduced angiogenesis and VEGF gene expression), as well as the inhibition of cell proliferation in various stages of colon adenocarcinoma in normoxia [2].
Aim: Investigation of the *in vitro* activity of AKG to human osteosarcoma cell line.
Materials and methods: Saos-2 (osteosarcoma) and hFOB 1.19 (normal osteoblasts) cell lines (ATCC), and the α-ketoglutaric acid disodium salt dihydrate were used in this study. The effect of AKG on the hFOB 1.19 and Saos-2 cells viability (MTT and NR uptake assay) was investigated. The effect of AKG on osteosarcoma cell proliferation (MTT assay) and migration (scratch assay) was examined. The influence of AKG on Saos-2 cell differentiation was evaluated by quantitative assay of cellular alkaline phosphatase (ALP) activity. The degree of mineralization of osteosarcoma's extracellular matrix (after AKG treatment) was evaluated by Alizarin Red S staining and by measuring the absorbance of the extracted dye. Statistical analysis of all data was carried out using unpaired *t* test.
Results: We observed that AKG did not exhibit significant effect on normal and osteosarcoma osteoblasts viability at the concentrations 1–50 mM. The non-toxic concentrations (10–50 mM) significantly inhibited Saos-2 cell proliferation and migration. None of the non-toxic concentrations influenced the activity of ALP, however all of them significantly reduced extracellular calcium deposits comparing to the control.
Conclusion: The obtained results suggests that AKG shows anti-osteosarcoma effect and may be useful as a chemopreventive agent.

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P15.60

Deficiency of microRNA-378 impairs revascularization after hind limb ischemia

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It is suggested that in ischemic/injured muscle tissue the formation of new blood vessels and muscle fibers take place simultaneously, as the regeneration of damaged muscle is associated with its revascularization. Recently it was reported that microRNA-378 (miR-378) may be involved in skeletal muscle differentiation. On the other hand its role in tumorigenesis, tumor growth and tumor vascularization was revealed. Nonetheless, so far no data are published concerning the involvement of miR-378 in regenerative neovascularization. To test such a hypothesis we used murine model of hind limb ischemia (HLI).
Femoral artery ligation (FAL) was performed in miR-378-deficient (miR-378-/-) mice and wild type (WT) mice. In response to HLI the restoration of the blood flow, measured using laser Doppler, was inhibited in miR-378-/- mice in comparison to WT mice. The number of necrotic toes was higher in miR378-/- animals and the extent of inflammatory response was stronger in the ischemic muscles of miR378-/- mice in comparison to WT mice. Namely, we observed enhanced number of inflammatory cells in ischemic muscles of both WT and miR-378-/- mice, with the effect of the latter being more prominent especially at day 1 and 21 after FAL. In accordance with that, we observed enhanced expression of interleukin-1β (IL-1β) in miR-378-deficient injured tissue. Moreover, the level of angiopoietin-2 (Ang-2), which may exert anti-angiogenic effects, significantly increased in response to HLI in miR-378-/- mice. To further analyze the role of miR-378 we performed flow cytometry analysis of the mobilization of proangiogenic cells (PACs) to peripheral blood in response to HLI. We demonstrated that PAC population characterized as Sca1+/CD117+/CD45-/CD31+/Tie2+/CD34+ tends to decrease in miR-378-/- mice at day 1 and day 3 after surgery in comparison to WT mice.
Taken together, our results suggest the significance of miR-378 for regenerative neovascularization of the ischemic tissues. Reported by us diminished restoration of blood flow in miR-378-/- mice may be related to higher inflammatory response, enhanced IL-1β and Ang-2 in ischemic muscles as well as decreased percentage of PACs in the peripheral blood.

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P15.61

Transcriptional infidelity of T7 RNA polymerase bypass of InDel mutations and causes unexpected phenotypic changes

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DNA–dependent T7 RNA polymerase is the most powerful tool for gene expression and in vitro RNA transcription as well. However, depending on the specific application, the quality of synthesized mRNA is more or less importance. Using NGS approach we analyzed the polymorphism of T7 RNA polymerase-generated mRNA of *mboIIM2* gene. It was found that the enzyme shows relatively high level of template-dependent transcriptional infidelity. The misincorporations and multiple insertions in A- and T-reach tracts of homopolymers in mRNA cause the epigenetic changes resulting in site-dependent rescue of a single and even double InDel frameshift mutants. Arising mixture of functional and non-functional protein variants creates a heterogeneous pool of proteins of almost identical molecular mass, which are indistinguishable from each other upon standard analysis.

P15.62

Stem cell-derived microvesicles carrying designer nucleases as novel tools for genome editing

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Microvesicles (MVs) are small particles originating from a cell membrane, which are released during cellular activation, apoptosis or in steady state. MVs are involved in cellular communication by transferring proteins, lipids and RNA into other cells. This capability can further be explored for delivery of specific bioactive components, including DNA-modifying enzymes. Zinc-finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALEN) and RNA-Guided Endonucleases (RGENs) constitute novel tools in genome modification and regulation. These site-specific nucleases induce DNA double strand breaks in a predefined locus and harness cellular DNA-repair machinery for the break repair, thus can be used to facilitate efficient genome editing.
The aim of this study was to investigate whether MVs isolated from Umbilical Cord-derived Mesenchymal Stem Cells (UC-MSC) are able to transfer active designer nucleases to other cell types, leading to specific genome modification.
UC-MSC were cultured in DMEM/F12 with 5% FBS. In a proof-of-concept study, UC-MSCs were transduced with a lentiviral vector encoding Green Fluorescent Protein (GFP). The efficiency was confirmed by FACS analysis. MVs were isolated from UC-MSC-GFP cells by ultracentrifugation, 100000g for 1h/twice. mRNA analysis confirmed the presence of GFP transcripts in UC-MSC-GFP-MVs. In the same way, MVs derived from UC-MSC-ZFN, UC-MSC-TALEN and UC-MSC-RGENs were obtained and characterized. In the next step, transfer assay of a nuclease-containing-MVs to target cells will be performed. This study suggests that transfer of genetic material via stem cell-derived MVs can be a powerful way to influence cell fate and can be explored for genome engineering purposes.

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