Student abstracts

P1

THERMODYNAMICS OF OPPOSITELY CHARGED BIOPOLYMERS

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Coacervation is a phenomenon in which a macromolecular aqueous solution separates into two immiscible liquid phases, i.e. the more dense phase is colloid-rich (the coacervate), and is in equilibrium with the relatively dilute colloid-poor phase. This liquid-liquid phase separation technique has been extensively used in cosmetic formulations and pharmaceutical microencapsulations. Phase separation into coacervates also occurs following the interaction of oppositely charged biopolymers: hyaluronic acid-chitosan system. [1-2].

Isothermal titration calorimetry (ITC) has been widely used for the characterization of polymer/ligand and complexes between oppositely charged species. Exclusively, there are only a few studies on the use of ITC for complex formation between oppositely charged polyelectrolytes. ITC is now routinely used to directly characterize the thermodynamics of biopolymer binding interactions. [3-4].

Our purpose here is to use ITC to increase our understanding of the complex formation between oppositely charged hyaluronic acid and chitosan. The interaction between chitosan (a weak polycation) and hyaluronic acid (a weak polyanion) was studied by ITC to obtain information about binding energetics, thermodynamics and stoichiometry of the interactions present in the system. All experiments were performed at 25 °C using a GE Healthcare (Microcal) VP-ITC instrument. The experiments were carried out at different ionic strengths to determine the effect of salt concentration on the binding process. At constant salt concentration, change in the ratio of hyaluronic acid to chitosan followed a non-monotonic behaviour. At constant hyaluronic acid to chitosan ratio (by mole), increase in salt concentration resulted as lower binding constants indicating the effect of charge screening. Positive entropy values indicate that the interaction is entropically driven due to counterion release. Positive enthalpy values indicate that the interaction is endothermic. In summary, hyaluronic acid-chitosan interaction is both enthalpically and entropically driven.

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P2

SULFONAMIDE LIGAND BINDING TO HUMAN CARBONIC ANHYDRASES

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Carbonic anhydrases (CAs) are metalloenzymes that catalyse the conversion between carbon dioxide and bicarbonate [1]. These proteins are involved in many physiological and pathological processes. Their inhibition with small molecule ligands can be applied for treatment of different diseases, such as glaucoma, cancer, obesity, epilepsy, osteoporosis, etc. There are nearly 30 small molecule ligands that are used as drugs for carbonic anhydrase related diseases. The main challenge for generation of new CA ligands is to attain specificity towards a selected CA isozyme, as there are 12 catalytically active CA isoforms in humans that have very similar structure.

During sulfonamide ligand binding to carbonic anhydrase at least four different reactions takes place [2]: 1) binding of deprotonated sulfonamide ligand to carbonic anhydrase when water molecule is coordinated to Zn^{2+} ion in its active center; 2) protonation of Zn^{2+} coordinated hydroxide ion; 3) deprotonation of ligand sulfonamide group; 4) compensating protonation or deprotonation of buffer. ITC or other biophysical methods measure the sum of all these reactions. However, for the QSAR analysis, only the intrinsic parameters (K_b, H) are relevant.

By using observed experimental parameters and employing thermodynamic additivity methods, it is possible to dissect the intrinsic binding parameters that do not depend

on linked reactions or applied reaction conditions [3]. One such example of the dissection, sulfonamide ligand binding to human carbonic anhydrase XIII, is presented here.

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P3

STRUCTURAL AND FUNCTIONAL STUDIES OF FICD AND ITS SUBSTRATE BIP

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The unfolded protein response (UPR) is initiated upon endoplasmic reticulum (ER) stress that leads to the accumulation of misfolded proteins in the ER. This homeostatic response activates signalling pathways that seek to reinstate a proper ER protein folding balance or eventually induces apoptosis if ER stress persists. Recently, we identified human FICD (Filamentation induced by cyclic AMP domain-containing protein) as a new UPR target [1]. This finding was corroborated by a recently published study [2]. FICD, which is the focus of this project, contains 458 amino acids comprising a predicted transmembrane (TM) domain followed by two tetratricopeptide repeat (TPR) regions. TPR motif has a basic helix-turn-helix fold and is typically involved in protein-protein interactions [3]. In addition, FICD is the only human protein known to contain a so-called FIC (filamentation induced by cyclic adenosine monophosphate) domain.

FICD protein is able to carry out a post-translational modification called AMPylation or adenylylation. This modification is defined as the stable and reversible covalent addition of an adenosine monophosphate (AMP) group to a hydroxyl side chain of a protein. Our current work has shown that immunoglobulin-binding protein (BiP), which is a key mediator of the UPR, is a bonafide target of FICD-mediated AMPylation in ER-derived microsomes.

In initial experiments of pull down, FICD was able to form an interaction with the BiP protein. By MicroScale Thermophoresis (MST), we found that BiP bound to full-length FICD-WT with a binding affinity around K_D = 1 M. These are still preliminary data and we intend to use also the FICD mutant with hyperactivity for AMPylation to interact with the BiP protein. In addition to the affinity determinations, we will investigate the thermodynamics of the binding between FICD and BiP, since FICD also has the potential to AMPylates itself and the link between auto-AMPylation and target AMPylation is still unknown in humans. In bacteria, it's known that the NmFIC-auto-AMPylation (FIC protein in *Neisseria meningitides*) is an essential process and earlier than the target AMPylation and this latter has an influence of the tetramerization mechanism of NmFIC [4].

Moreover, we intend to investigate the role of the TPR domains of FICD in the interaction with BiP. For that, we have a construct of FICD that lacks the TPR domains and it can be used for binding assays and further comparison with the full-length FICD. In addition, we made a construct that contains only the TPR region and a partial linker region aiming to understand the TPR's role in the structure of the FICD protein. We hypothesize that the partial linker region could works as a groove to the ligand binding due to the recent work that solved the partial crystal structure of FICD [5]. Here, we expect to disclose the mechanism involving FICD and its substrate BiP aiming to understand the FICD's role in the UPR system and how the AMPylation can influence BiP function as a chaperone.

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P4

NEW HYBRIDS OF TETRAHYDROACRIDINE DERIVATIVES AS A ACETYLCHOLINESTERASE WITH MULTIFUNCTIONAL ACTIVITY

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Neurodegenerative disorders are characterized by the progressive dysfunction of the central nervous system (CNS), mostly resulting from the loss of CNS neurons. The most prevalent neurodegenerative conditions include Alzheimer's disease, Parkinson's disease, Huntington's disease, as well as prion diseases; however, Alzheimer's disease corresponds to about 70% of those disorders. Acetylcholinesterase inhibitors, referred to as the first line of therapy, constitute the principal group of agents used in the therapy of Alzheimer's disease.

9-amino-1,2,3,4-tetrahydroacridine, referred to as tacrine, is a reversible acetylcholinesterase inhibitor. It was described in 1961 for the first time, and in 1993 U.S. FDA registered it as the first agent used in the causative treatment of Alzheimer's disease. Despite its great therapeutic effect, it is highly toxic to hepatocytes and have a number of other adverse effects. One of the ideas that can help overcome adverse effects of tacrine without losing its activity is hybrids. They constitute a novel group of tacrine derivatives; the role of these compounds is complex. Their structure is similar to that of dimers, but the second molecule is a substance characterized by different structure or effect rather than the tetrahydroacridine derivative itself. [1, 2] Such modification would improve the selectivity to cholinesterases and decrease toxicity on one hand, and exert positive effect on Alzheimer's disease-associated processes on the other.

The principal aim of hereby presented research is to modify the basic structure, i.e. the tetrahydroacridine compound. The targeted hybrids were obtained as a result of 1,2,3,4-tetrahydroacridine coupling with other tacrine derivatives. An aliphatic chain with various number of carbon atoms is the linker between these two moieties. We expect the synthesized compounds to have better ADME and TOX profile comparing with tetrahydroacridine itself. After the synthesis, in silico tests were made to confirm the expected mechanism of action. 3D-molecular docking of the most promising compounds to the active site of AChE and BuChE and pharmacophore modeling were held using SCIGRESS, molecular design modeling software developed by Fujitsu. [3] The obtained results let us predict the activity of the synthesized compounds and may be used in further QSAR studies. Nevertheless, the predictive power of the constructed model must be estimated during further biological studies.

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P5

MULTIFUNCTIONAL ACTIVITY OF NEW TETRAHYDROACRIDINE DERIVATIVES AS ACETYLCHOLINESTERASE INHIBITORS

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Alzheimer's disease (AD – Alzheimer's disease) is a progressive disease that is the most common form of dementia. This disease belongs to neurodegenerative fatal disorders and affects mainly elderly people. AD affects about 6% of the population in the world, aged over 65. Disease increases dramatically with age being basic risk factor. It is characterized by memory loss, progressive cognitive decline, and behavior changes caused by reduced levels of the neurotransmitter acetylcholine. Despite dynamic progress in science, both in nanotechnology and biotechnology, the effective methods of early detection of Alzheimer's disease, it is still incurable. There are only four acetylcholinesterase inhibitors approved by Food and Drug Administration so far. They are regarded to one of the most important methods in AD treatment. First drug approved was tacrine (THA, 9-amino-1,2,3,4-tetrahydroacridine). It is the strongest and most effective acetylcholinesterase inhibitor, but it interacts with many other drugs and causes a lot of side effects. Therefore it was withdrawn from the market due to its hepatotoxicity. During the last decades scientists put a lot of effort in AChEIs (AChEIs - acetylcholinesterase inhibitors) modifications and attempt to find new structures. Unfortunately there is still no effective method in AD therapy. Moreover, we still don't know the exact cause of that disease. There are a few theories about the AD occurrence mechanisms but despite intensive studies it wasn't possible to develop effective drugs without serious side effects. Due to evergrowing number of AD patients, intensive research of the innovative therapies is highly recommended.

The main goal of the project is to develop new hybrids of the tetrahydroacridine and identify their potential for neurodegenerative disease therapy. Analysis of their AChE and BuChE inhibition potential will be made by different *in vitro* methods, including beta-amyloid aggregation, cytotoxicity and of the pharmacokinetic and pharmacodynamics properties of the obtained compounds using methods of computational chemistry. The synthesis of new, active compounds can also be useful for further research and development of the potential drugs for neurodegenerative disease therapy.

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P6

VIRTUAL SCREENING AND STRUCTURAL STUDIES OF HMGB1 INHIBITORS

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The prominent role of a Damage-associated Molecular Patterns (DAMPs) protein, namely the High Mobility Group Box-1 (HMGB1), in the non-infectious inflammatory response of several diseases (myocardial infarction, sepsis, autoimmunity, atherosclerosis and cancer) lately emerged with increasing impact. In particular, it has been recently reported, that the inhibition of the HMGB1 inflammatory axe remarkably reduces the motility and growth of malignant mesothelioma (MM) as well as the metastatic progression of melanoma [1].

At this respect, we identified, by a combination of computational studies and experimental evidences, the natural compound glycyrrhizin as selective HMGB1 inhibitor of its cytokine-releasing activity, although with a high dissociation constant (K_d) of ~150 ?M [2]. Additionally, Salicylic Acid (SA) and its derivative Amorfrutin were discovered to weakly bind HMGB1 (Kd in high micromolar range) and work as inhibitors of the extracellular functions of HMGB1 (IC_{50}~5-10 ?M [3]). SA was also shown to delay MM growth by xenograft mice

and significantly improved survival of treated animals. Starting from these reference compounds, we are currently investigating further this promising as relatively unexplored HMGB1 path by identifying new and more potent inhibitors to interfere with the metastatic progression of types of cancer with inflammation-induced phenotypic plasticity.

In a multidisciplinary approach, we started with a virtual screening of a large ZINC database of compounds and shortlisted a set of plausible ligands. Eight different chemical cores (**C1-C8**) were identified and used to clusterize a reduced set of leads. We tested representative molecules of **C1**, **C6** and **C8**, recording their ability to inhibit HMGB1 cytokine activity in cell migration assays, with IC_{50} of 10, 50 and 70 ?M respectively. Most importantly, a very promising hit emerged from **C7**, represented by *5,5'-Methylenedi-2,3-cresotic acid (MCA)* that displayed an inhibitory effect of HMGB1 chemotaxis activity in the submicromolar range, better than any other HMGB1 inhibitor so far. Hence, a preliminary characterization by receptor- (15N HSQC) and ligand-based (STD, waterLOGSY) NMR techniques is here presented focusing our attention on the MCA-HMGB1 interaction.

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P7

INTERACTION BETWEEN DIFFERENT PHENOLIC COMPOUNDS AND PDIA3

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Flavonoids, plant secondary metabolites present in fruits, vegetables and products such as tea and red wine, show antioxidant, anti-inflammatory, antithrombotic, antiviral and antitumor activity [1]. Punicalagin and ellagic acid are polyphenolic compounds, isolated from pomegranate fruit. They have shown remarkable pharmacological activities including anti-inflammatory, hepatoprotective and antigenotoxic activities. Other potential applications include infant brain ischemia, male infertility, Alzheimer's disease, arthritis, and obesity [2]. PDIA3, a member of the protein disulfide isomerase family, is an ER protein mainly involved in the correct folding of newly synthetized glicoproteins. This protein is associated with different human diseases such as cancer, prion disorders, Alzheimer's and Parkinson's diseases and it has the potential to be a pharmacological target [3]. In this study the interaction of different flavonoid, as well as pomegranate extracts, with PDIA3 and their effects on protein reductase activity were evaluated. The interaction was investigated by quenching analysis of protein intrinsic fluorescence. This analysis was extended to the PDIA3 a' domain, and the effect of flavonoids on the DNA binding properties of a' domain was evaluated by EMSA analysis. A higher affinity for PDIA3 was observed for flavonoids with an intermediate hydrophilicity degree (1-3 hydroxyl groups) which also inhibit disulphide reductase activity of the PDIA3, but do not significantly affect the DNA binding activity of the a' domain. Probably this interaction involves a different binding site from that of green tea catechins, which, as previously demonstrated, can bind to the a' domain and decrease its interaction with DNA [4]. Different from all the other flavonoids analyzed punicalagin, but not ellagic acid, showed a marked inhibitory effects on protein reductase activity as well as a good binding affinity. On the basis of these observations punicalagin seem to be a promising compound to develop specific inhibitors for PDIA3.

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P8

STRUCTURAL AND FUNCTIONAL STUDIES OF THE TRANSLOCATOR PROTEIN

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The translocator protein (TSPO) is a 18kDa protein with five alpha-helical transmembrane domains. It is located in the outer mitochondrial membrane of steroid-synthesising cells and is of pharmacological interest as it is used both as a biomarker and a therapeutic target. It has been associated with a number of pathological conditions such neurodegenerative diseases and cancer and is proposed to be involved in cholesterol transport through the mitochondrial membrane; possibly together with the steroidogenic acute regulatory protein (StAR). Several structures of bacterial TSPO homologues have been published so far, but nevertheless there are a number of open questions regarding TSPO structure and function, such as the fold of the eukaryotic TSPO homologues and the relevance of the oligomerisation state for activity, as well as how TSPO interacts with its substrates and binding partners.

The aim of this project is to gain a better understanding of the function and fold of TSPO using structural, biophysical and biochemical methods, along with obtaining a high-resolution crystal structure of a eukaryotic TSPO homologue.

So far, different constructs of 12 eukaryotic TSPO homologues have been expressed in small scale in HEK cells. Expression levels and homogeneity of the expressed proteins were analysed using FSEC, and their quality was characterised by radioligand binding and thermostability assays. The most promising constructs were chosen for large scale expression in suspension culture by induction of stably transfected cells. The purified protein will be used for interaction studies with ligands and potential binding partners (such as StAR), biophysical characterisation and crystallisation trials. Preliminary results are very promising and show that the established expression and purification protocol will result in sufficient amount of protein to conduct structural studies and gain mechanistic insight into this family of proteins.

P9

EXPLORING THE LINK BETWEEN CATALYSIS AND REGULATION OF A KEY LIPID BIOSYNTHETIC ENZYME FROM THE MALARIA PARASITE

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Despite ongoing antimalarial research, malaria is still one of the most serious infectious diseases, with the vast majority of death cases caused by *Plasmodium falciparum*. Emergence of multidrug-resistant parasite strains urges the identification of antimalarials with a novel mechanism of action [1]. Accordingly, *de novo* phosphatidylcholine biosynthesis of the parasite was recognized as a validated antimalarial target [2]. Within this pathway, CTP:phosphocholine cytidylyltransferase (*Pf*CCT) was shown to catalyze a rate-limiting step [3, 4].

The understanding of the molecular mechanism of the *Pf*CCT regulation stands in the focus of our interest. Specifically, we analyze the role of the E helix segment located at the C-terminal of the catalytic domain in the enzymatic cycle. Importantly, this region was proposed to be involved in the lipid-induced regulatory action of the enzyme as it provides essential contact to the reversible membrane binding segment of CCT enzymes [5]. We engineered catalytic domain constructs of *Pf*CCT with a series of truncations at its C-terminal áE helix and characterized their biological function with a combination of enzyme activity assays and ligand binding measurements with ITC and tryptophan fluorescence. We found that the truncation caused inactivation of *Pf*CCT as well as a severe attenuation of its CTP substrate binding capability. Nevertheless, a stepwise elongation of the C-terminal part including the E helix and the subsequent nonconserved region restored the catalytic function of the enzyme. Insights from the recently solved crystal structure data of the truncated *Pf*CCT construct (E. Guca et al. 2016, to be published) revealed the steric displacement of two conserved threonine residues within the E helix could explain the observed perturbation of enzymatic function. Indeed, we verified the critical

role of these threonines by biochemical characterization of their alanine point mutants.

Our further aims are to engineer a construct containing the membrane binding domain of this enzyme and discover the membrane lipid-protein interaction in order to gain a comprehensive picture of the interplay between catalysis and regulatory mechanism with relevance for the whole CCT enzyme family. The prospect of the well-defined role of these key regulatory segments of the enzyme could be further exploited for more specific drug targeting.

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P10

IDENTIFICATION OF TRAIL-BINDING AND -INHIBITING PEPTIDES

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Novel approaches to sepsis therapy make use of targeted inhibition of specific signalling molecules such as TNF, which would result in modulation of the immune response and finally in sepsis symptom relief [1]. The signalling molecule TNF-related apoptosis inducing ligand (TRAIL, TNFSF10) has been reported to be crucially involved in establishing the immunoparalysis by suppressing the adaptive immune response as well as to be involved in tissue damage [2]. We would expect TRAIL-inhibiting substances to reinforce the adaptive immunity in the course of sepsis leading to an improved control of secondary infections. Moreover, TRAIL inhibition should result in reduced tissue damage.

TRAIL is a member of the TNF superfamily, and as such is a type II membrane protein that is processed proteolytically at the cell surface to form a soluble ligand (residues 114–281). Its extracellular domain is highly homologous to that of tumour necrosis factor (TNF) and FAS ligand (CD95, FASLG, TNFSF6). TRAIL can bind two apoptosis-inducing receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and additionally cell-bound receptors incapable of transmitting an apoptotic signal, the decoy receptors TRAIL-R3 (LIT, DcR1) and TRAIL-R4 (TRUNDD, DcR2). Finally, the soluble receptor osteoprotegerin (OPG) has also been shown to be capable of binding to TRAIL [3].

In this project we follow different approaches including phage display, yeast-2-hybrid system as well as *in silico* design and screening approaches to identify TRAIL- or TRAIL-receptor binding peptides. Based on an alanin-screen of TRAIL published by Hymowitz *et al.* [4] we have chosen region for the virtual design/screening approach. Four peptides resulting from the virtual modelling shall be tested for binding to DR5, and/or for interfering with the binding of TRAIL to its cognate receptors. They are currently tested in a biological system to investigate the peptide-mediated interference with TRAIL-induced apoptosis.

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P11

SCREENING AND QUANTITATION OF GALECTIN-3C CARBOHYDRATE LIGANDS

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Galectins represent an important multifunctional class of animal lectins, playing roles in various biological processes, such as inflammation, immunity and cancer. They can contain one or two carbohydrate recognition domains (CRDs), responsible for binding of -galactosides. In affinity mass spectrometry study (AMS) we used the C-terminal fragment of the recombinant human galectin-3.

AMS is a tool that is gaining popularity for studying protein-ligand interactions. Its main advantages are speed, sensitivity, as well as the ability to determine the stoichiometry of macromolecular complexes. However, the accuracy of the data obtained with AMS often depends on the system studied, complexity of ligand mixtures tested, as well as the nature of the galectin-ligand interaction. For example, we found that while some ligands do not exhibit significant variability in galectin binding based on its charge state, others may do so. The N-glycans tested showed significantly higher degree of non-specific binding with increased charge state of Gal-3C. The quantitative AMS data analysis showed that the Kd values obtained are in good agreement with the data available in the literature. However, due to lack of ITC (isothermal titration calorimetry) data for most ligands, it is difficult to estimate the accuracy of AMS experiments.

While validation, as well as optimization of AMS studies represents an active field of research, fluorescence polarization (FP) assay represents an alternate method for quick measurements of Kd values for a series of carbohydrate ligands. Since the fluorescent tag often contributes to galectin-carbohydrate binding interactions, competitive FP assays are preferred. While ITC represents a method of choice for accurate measurement of Kd values for the galectins, it requires much bigger amounts of analytes, and is not suitable for large screening studies. Thermophoresis represents a possible alternative for FP assays, with bonus ability to perform the binding studies under physiological temperature conditions. Finally, while surface plasmon resonance (SPR) studies are not suitable for measuring galectin-carbohydrate interactions, they are well suited for studying protein-protein interaction properties of the galectins, including their oligomerization phenomena.

P12

FLUORESCENCE ANALYSIS FOR COMPETITIVE BINDING OF GLICLAZIDE AND QUERCETIN ON HUMAN SERUM ALBUMIN

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Co-administration of a synthetic drug and a compound of natural origin is common for patients, who combine their pharmacotherapy using various dietary supplements. Many of them often contain natural polyphenols. The effect of co-administration is not completely clear. The competitive binding of compounds can cause a decrease in the amount of drug bound to protein and increase the biological active fraction of the drug.

The aim of this study was to analyse simultaneous interactions of a common flavonoid quercetin (Q) and the antidiabetic drug gliclazide (G) with human serum albumin (HSA) in phosphate buffer (pH=7.4). Fluorescence analysis was used to determine the binding and quenching properties of HSA–ligand complexes in binary and ternary systems. The association constants (K_a) of systems were determined with the use of Scatchard analysis. The quenching (K_Q) constants were determined on the basis of the modified Stern-Volmer equation. Fluorescence quenching mechanism of HSA–drug complexes both was suggested as static quenching. The influence of Q on the complex of G with HSA has been described for the binary and ternary systems by the quenching curves and binding constants comparison. The effect of drugs on the conformation of HSA was analysed using synchronous fluorescence spectroscopy.

Fluorescence data analysis showed that the presence of Q hindered the interaction between HSA and G, as the binding constant for G in the ternary system was remarkably lower compared to the binding constant of the binary system. This indicates a possibility for an increase of non-bound fraction of G and can lead to more significant hypoglycemic effect of G, what can be considered as an undesired adverse effect.

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P13

CHARACTERIZATION OF INTERACTIONS BETWEEN PROTEINS ISOLATED FROM *CANDIDA* SPP. CELL WALL AND HUMAN PROTEINS INVOLVED IN INFLAMMATORY RESPONSE AND INNATE IMMUNITY

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Pathogens produce a wide range of molecules called virulence factors, that allow them to penetrate into a host organism, combat the immune system and which facilitate acquisition of nutrients and further spread. Among the major virulence factors of pathogenic fungi are adhesive proteins exposed at their cell surface. The primary role of adhesins is binding of host proteins, including those involved in the defense mechanisms, such as components of plasma proteolytic cascades, antimicrobial peptides or proteins associated with neutrophil extracellular traps (NETs). Therefore, pathogens might hijack and affect the host systems involved in maintaining homeostasis and possess the ability to evade host immune response [1].

During work in our research team, we identified with the use of affinity chromatography and chemical cross-linking several proteins derived from the cell walls of the major fungal pathogens of humans — *Candida* spp. — that bind to components of proinflammatory plasma contact system [2], NET-associated proteins and antimicrobial peptide – cathelicidin LL-37. Within this group, there were both typical adhesins covalently bound to the fungal cell wall via GPI anchor, such as agglutinin-like sequence (Als) proteins, as well as atypical proteins, more loosely associated with fungal cell surface, including enolase, phosphoglycerate mutase, 6-phosphogluconate dehydrogenase or triosephosphate isomerase.

The interactions between individual pairs of purified candidal protein and host protein were tested with microplate enzyme-linked ligand sorbent assays, and then characterized kinetically and thermodynamically with the surface plasmon resonance measurements [3]. Hence, another challenge is to corroborate obtained data using additional advanced methods in order to confirm the important role of particular fungal proteins in interactions with selected host molecules involved in innate immunity. Such advanced methods will also be applied for studying molecular mechanisms of fungal pathogenesis that are related with binding and activation of components of host fibrinolytic system, including human plasminogen and its activators.

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P14

INVESTIGATING COLD SHOCK PROTEIN INTERACTIONS WITH SINGLE STRANDED DNA USING SINGLE MOLECULE FORCE SPECTROSCOPY

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Single-molecule force spectroscopy (SMFS) is a technique that provides a direct mechanical measurement of molecular forces caused by a single protein unfolding [1] or a complex unbinding [2]. SMFS techniques can be applied to the investigation of protein-DNA interactions by (i) measuring the impact of single stranded DNA (ssDNA) binding on the mechanical stability of a protein and (ii) measuring the unbinding force of ssDNA-protein complexes. These measurements can be complemented with experiments on the binding energies and kinetics to explore the link between the single molecule and bulk studies. Greater understanding of the mechanism of binding can be gained using molecular dynamics simulations. Combining these methods will enhance the investigation into the impact that binding has on the stability and dynamics of each macromolecule.

The cold shock protein (CSP) from the bacterium *Bacillus subtillis*, *Bs*CSP was chosen as a good candidate to investigate ssDNA-protein interactions as previous studies in our group have demonstrated that *Bs*CSP has a clear mechanical fingerprint in SMFS experiments [3]. *Bs*CSP regulates biological functions and is key for adapting systems to cold shock [4]. The function of CSPs is therefore highly temperature specific and involves the binding of nucleic acids over a temperature range that depends on the natural environment of the organism. The effect of ssDNA binding on the mechanical stability of the BsCSP has been measured at different temperatures, showing an increase in the force required to unfold the protein of up to 50% or 20 pN.

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P15

GAPDH INTERACTS WITH ABASIC SITES IN DNA: A NEW PUTATIVE FUNCTION OF A WELL-KNOWN ENZYME

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an evolutionary conservative and abundant protein widely known as a glycolytic enzyme. Active GAPDH is a tetramer consisting of identical 37 kDa-subunits. Interestingly, GAPDH has many additional putative functions: it takes part in membrane transport, microtubule bundling, signal transduction and many other processes. GAPDH is known to interact with DNA and RNA, some types of DNA damages and DNA repair enzymes (APE1, HMGB1).

Abasic (AP) sites are among the most common genomic DNA lesions. The loss of DNA bases and the subsequent formation of AP sites occur as a result of the spontaneous hydrolysis of the *N*-glycosidic bond or the removal of the damaged bases at the early stage of base excision repair, which is catalyzed by DNA glycosylases. Unrepaired AP sites are mutagenic and cytotoxic.

Certain proteins can interact with the deoxyribose of an AP site to form a Schiff base, which can be stabilized by borohydride treatment. Several types of AP DNA were used to trap proteins in human cell extracts by this method. In the case of single-stranded AP DNA and the AP DNA duplex with both 5'- and 3'-protruding ends, the major crosslinking product had an apparent molecular mass of 45 kDa. Using peptide mass mapping based on mass-spectrometry data, we identified the protein forming this adduct as an isoform of GAPDH. Next, we have studied interaction of GAPDH purified from HeLa cells with different AP DNAs. Intriguingly, about 30% of GAPDH–AP DNA adducts were borohydride-independent. These data indicate that GAPDH, at least partially, may be covalently linked with an AP site by a mechanism other than the Schiff base formation. Indeed, GAPDH was shown to lose the ability to form adducts with AP DNA after disulfide bond reduc-

tion. NAD^+ also inhibited GAPDH–AP DNA adduct formation. GAPDH was proven to crosslink preferentially to AP DNA cleaved via the -elimination mechanism (spontaneously or by AP lyases), but it did not display the AP lyase activity. Moreover, we used AP DNA as a probe for GAPDH detection in cell extracts.

According to literature data, under oxidative stress GAPDH undergoes disulfide bond formation that results in the enhancement of its DNA-binding capacity and is translocated to the nucleus. At the same time, oxidative DNA damages lead to the activation of PARP-1, which synthesizes poly(ADP-ribose) using NAD^+. As a result, the pool of NAD^+ is exhausted and the NAD^+-binding site of GAPDH is empty. Thus, the enzyme acquires the ability to bind DNA and can be trapped in a stable covalent complex with an unrepaired AP site that would hamper DNA repair. This may be a suicidal event in the case of multiple DNA damage that could be one of the factors leading to cell death.

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P16

KINETIC STUDIES OF MELANOCORTIN-4 RECEPTORS USING FLUORESCENCE ANISOTROPY

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The melanocortin-4 (MC_4) receptors are important drug targets as they regulate energy homeostasis, eating behavior and sexual functions. The ligand binding process to these G protein-coupled receptors is subject to considerable complexity. The different steps of the complex dynamic regulation could be characterized by ligand binding kinetics [1]. Optimization of these kinetic parameters in terms of on-rate and residence time can increase rapid onset of drug action and reduce off-target effects [2].

There are a number of homogeneous fluorescence-based assays that allow continuous online monitoring of ligand binding kinetics. Among them, fluorescence anisotropy (FA) has been implemented for the kinetic studies of MC_4 receptors expressed on budded baculoviruses [3, 4]. However the slow dissociation of the used fluorescently labelled peptide NDP- -MSH does not enable to reach equilibrium. To overcome this problem, two novel red-shifted fluorescent ligands were designed. These cyclized heptapeptide derivatives were referred to as UTBC101 and UTBC102.

Both of these ligands exhibited nanomolar level affinity towards MC_4 receptors, but had relatively different kinetic properties. The dissociation half-lives of UTBC101 (= 151 min) and UTBC102 (= 8 min) were shorter compared to the previously reported Cy3B-NDP- -MSH (= 224 min) [3]. The significantly shorter dissociation half-life of UTBC102 enables to achieve equilibrium in screening assays, whereas the higher affinity of UTBC101 helps to resolve a wider range of competitor potencies. In summary, these two ligands could potentially complement each other in the on-rate and residence time screening of unlabeled ligands.

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P17

TARGETING CATHEPSIN B IN THE TUMOUR MICROENVIRONMENT BY INHIBITORY DARPINS

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Cathepsin B is a lysosomal cysteine protease involved in tumour cell invasiveness and angiogenesis . While normally the localization of the protease is confined to the endo-lysosomal vesicles, in tumours it is secreted to the membrane or the extracellular space by tumour as well as stromal cells, such as tumour-associated macrophages, fibroblasts and endothelial cells .

Pharmacological inhibition of cathepsin B by small-molecule inhibitors was shown to inhibit tumour growth and metastasis in animal models, and tumour-specific up-regulation of cathepsin B has been explored for diagnostic purposes as well as targeted drug delivery .

We propose that small protein binders such as DARPins offer a great opportunity for design of highly selective reversible cathepsin B inhibitors that could be applied with theranostic mode of action – as cathepsin protease inhibitors and as diagnostic imaging probes. These engineered proteins have several key characteristics that allow demanding chemical or biochemical modifications without affecting the binder activity, namely the small size, high stability and ease of site-specific labelling.

Here we present the selection and characterization of two inhibitory DARPins with high affinities for human and mouse cathepsin B and no detectable affinity for highly homologous cysteine cathepsins. We used a combination of competition assays and enzyme kinetic studies to characterize the binding, and we confirmed the results with solved crystal structures of the complexes. Furthermore, both DARPins successfully inhibited cathepsin B in human and mouse cancer cell lines, which suggests they are suitable candidates for further therapeutic and diagnostic imaging development.

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P18

A GENERIC PROTOCOL FOR THE CHARACTERIZATION OF PROTEIN SMALL MOLECULE COMPLEXES USING AFFINITY-TAGGED PROTEINS SHEDS NOVEL LIGHT ON NDPK1 AND CAMP INTERACTION IN PLANT CELLS

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Multiple biological processes are triggered by molecule-molecule interactions. These include interactions between proteins, proteins and nucleic acids, and proteins and small molecules. Discovery and characterization of the role of these complexes are objects of intensive investigation. In recent years, many new techniques allowing analysis of protein-small molecules interactions, also at "omics"-scale, became available .

We exploited protein-to-small molecule strategy based on affinity purification method. We adjusted previously

available yeast protocol and used IgG antibodies against chimeric proteins consisting of an IgG-binding domain fused to the protein of interest to unravel potential protein-small molecule complexes in native plant lysate . We examined small molecule ligands of three nucleoside-diphosphate kinase proteins (NDPK1-3). Primary role of these multifunctional proteins is to maintain nucleoside triphosphates (NTP) level. They transfer phosphate mainly from ATP to cognate nucleoside diphosphates (NDP) via ping-pong mechanism. NDPK proteins might play crucial role in signal transduction by elevating GTP level and thus, enabling activation of GTP-binding proteins .

Here we propose that NDPK1 but not NDPK2 and NDPK3, binds 3', 5' cyclic adenosine monophosphate (3',5' cAMP). 3'5'cAMP is an important second messenger, conveying cAMP-dependent pathway. Numerous vital animal processes are initiated by interaction between cAMP and cAMP-dependent kinases hence we suggest that our results open an exciting new paradigm on cAMP signaling in plants.

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P19

THYMIC STROMAL LYMPHOPOIETIN (TSLP) AND ITS RECEPTOR AS TARGETS FOR THE DEVELOPMENT OF ANTI-INFLAMMATORY INHIBITORY AGENTS

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Thymic Stromal Lymphopoietin (TSLP) is an interleukin-7-related cytokine expressed in epithelial cells and keratinocytes. It plays a central role in the pathology of inflammatory allergic disorders such as asthma, atopic rhinitis, as well as in other non-allergen induced conditions. The activated TSLP receptor (TSLPR) is formed by ligand-induced heterodimerisation out of the specific TSLP receptor alpha chain and the IL-7 receptor alpha chain and signals via the JAK/STAT pathway. Because of its involvement in various diseases, the TSLP/TSLPR system is a potentially interesting therapeutic target. [1]

We have explored possibilities to specifically block TSLP-induced receptor activation for the human cytokine (hTSLP) by means of (i) recombinant ligand binding receptor exodomains, (ii) functional antibodies to both receptors [2] and ligands and (iii) TSLP variants. These agents were analyzed for biological activities and inhibitory properties employing cellular models such as novel TSLP-responsive reporter cell lines.

Recombinant TSLPR exodomains proved as competitive inhibitors of TSLP activity. Monoclonal antibodies were isolated and able to block TSLPR activation and intracellular signaling. Based on structural considerations and mutational analysis, TSLP variants with antagonistic properties were identified. These approaches are systematically further extended and exploited.

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P20

EXPLORING THE NON-CATALYTIC REGIONS OF THE LYSINE SPECIFIC DEMETHYLASE FAMILY AS SITES FOR DRUG TARGETING

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The epigenetic system allows the cell maintains his homeostatic balance by controlling the chromatin structure of genes. Eventual alterations to such control systems establish changes in chromatin structure that are related to some diseases as cancer. In addition to DNA methylation and histone acetylation, recently the research has been very interested in understanding of histone methylation effect. Two enzyme families, lysine methyltransferases (KMTs) and demethylases (KDMs) control histone lysine methylation. KDMs encompass the Jumonji C (JmjC) domain–containing proteins and the Lysine specific demethylase (LSD) family. LSD1 was the first discovered

histone demethylase as reported independently by our and Shi's groups [1,2].

The enzyme is tightly associated to the co-repressor CoREST, forming the complex LSD1/CoREST, for which current literature describes at least 30 interactors, including transcription factors and non-histone substrates, many of them directly involved in cancer biology. The link between LSD1, tumor pathogenesis and the discovery that the monoamine oxidase (MAO) inhibitor tranylcypromine (TCPA) blocks LSD1 activity, represented the starting point for intensive drug discovery programs aimed at TCPA based irreversible active-site LSD1 inhibitors [3].

On the other hand, the existence of so many inter-macromolecular interactions finds a structural counterpart in characteristic non-catalytic domains, as sites for drug targeting.

The aim of the present project is to advance our knowledge on the characteristic non-catalytic domains of LSD1, in order to understand how they control substrate specificity (also with reference to non-histone substrates), contribute to the recognition of the nucleosomal particle, and interact with other proteins and RNAs. The main goal will be to discover and explore sites to be targeted by innovative inhibitors as opposed to the more "classic" active-site ligands.

Binding of the candidate compounds are experimentally verified by thermal shift assays, a methodology by which we screen candidate compounds that exert a stabilizing effect on proteins by measuring the unfolding temperature [4]. Moreover, the binding affinities of labelled/tagged peptides and oligonucleotides can be measured directly by probing the changes in fluorescence polarization. Then, to carry out a competition assay, untagged ligands competing with a fluorescent molecule for the same site are used. In this way there is the possibility to probe both active site and non-active site ligands.

These inhibitors can be expected to selectively target LSD1 only in the context of processes involving specific protein complexes and can be considered as innovative ligands for future epi-drug design and development.

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P21

PHYCOCYANOBILIN, A BIOACTIVE TETRAPYRROLIC COMPOUND OF MICROALGA *SPIRULINA*, BINDS WITH HIGH AFFINITY TO HUMAN SERUM ALBUMIN AND ENHANCES ITS STABILITY

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Human serum albumin (HSA) is an important regulator of the pharmacokinetic properties of bioactive compounds. Phycocyanobilin (PCB) is a blue tetrapyrrole chromophore of C-phycocyanin with proven health-promoting activities. The aim of our study was to examine binding of PCB for HSA and to investigate its effects on protein stability. Based on a computational approach, we demonstrated two putative high-affinity binding pockets on HSA of virtually identical energies (subdomains IB and IIA). Results obtained by protein and pigment fluorescence measurements, circular dichroism (CD), and bilirubin (structural analog of PCB)-displacement experiments confirmed high affinity (binding constant of 2.2 106 M-1), stereoselective binding of PCB M-conformer to HSA and its competition with warfarin (subdomain II A marker) and hemin (subdomain IB marker). Fluorescence and UV/VIS absorption spectra indicated that PCB underwent conformational change from cyclic to more stretched conformation, upon binding to HSA. CD and fluorescence melting curves of HSA in the presence of PCB showed increased thermal stability of HSA upon chromophore binding. Trypsin digestion study showed that HSA?PCB adduct was more resistant to proteolysis than free HSA. Fourier transform infrared spectroscopy and CD spectra have revealed slightly higher alpha-helical content in HSA?PCB adduct than in free protein. The present results provide valuable information for the transportation and distribution of PCB in vivo, which may be of importance for the understanding of its numerous beneficial effects, including partial stabilization of HSA as a consequence of PCB binding.

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P22

BINDING OF *TRICHINELLA SPIRALIS* MUSCLE LARVAE EXCRETORY-SECRETORY ANTIGENS TO DC-SIGN

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During the course of infection parasite *T. spiralis* communicates with the host organism and affects the cells of immune system through excretory-secretory products (ES L1) released from the muscle larvae. Previous results showed that *in vitro* stimulation of dendritic cells (DCs), key players in the polarization of immune response, with *T. spiralis* ES L1 antigens led to partial maturation of these cells [1], however, receptors on DCs involved in this interaction are not yet revealed. It has been shown that ES L1 antigens are highly glycosylated and, among others, decorated with high-mannose glycans [2], a structure that has a potential to be recognized by calcium dependent dendritic cell surface receptor DC-SIGN (dendritic cell-specific ICAM-3-grabbing non integrin). DC-SIGN is involved in recognition and binding of endogenous or exogenous antigens and in modulation of DC maturation status. In the current study, possible interaction between ES L1 and DC-SIGN was investigated using recombinant DC-SIGN-Fc (DC-SIGN fused at the COOH terminus to a human IgG1-Fc fragment). Binding of DC-SIGN-Fc to immobilized ES L1 antigens was detected in solid-phase assay - ELISA, and in western-blot. A chelator agent EDTA was used as inhibitor in order to confirm the specificity of this interaction.

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P23

INVESTIGATION AND CHARACTERISATION OF A STAPHYLOCOCCUS AUREUS ENZYME-INHIBITOR COMPLEX

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Staphylococcus aureus is a virulent pathogen that is currently the most common cause of infections in hospitalized patients. The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors. It becomes resistant rapidly against the commonly used antibiotic agents, thus investigation of its molecular processes is crucial in the development of new drugs and therapies.

Maintaining of DNA integrity against mutagenic effects and agents is important for the cell viability. For that purpose, the bacterial cell produces several common DNA repair enzymes that can act in cascade systems. The first member of the base excision repair system is an uracil-DNA glycosylase enzyme (UDG) which can remove the uracils in DNA that result from the spontaneous deamination of cytosine or the incorporation of dUTP during replication.

Interestingly the *S. aureus* genome encodes a recently described inhibitory protein of SaUDG, so-called *S. aureus* uracil-DNA glycosylase inhibitor (SaUGI) [1]. Previously only two uracil-DNA glycosylase inhibitors (PBS2 phage UGI and Bacillus phage p56 protein) have been described. SaUGI is therefore the third uracil-DNA glycosylase inhibitor that has been identified, and the first in a species other than bacterial phage.

In the present work we focused on characterization of the interaction between SaUDG and SaUGI. Our objective is to discover the important amino acids in binding, but only those which are the part of the natural diversity. For this aim, firstly we aligned naturally occurring SaUGIs and then analysed the amino acid sequence in the interaction surface. We made site-directed mutagenesis, and purified three mutant proteins, that showed different kinetical behaviour as compared to wild-type enzymes. In our research we applied several methods including thermofluorimetry, isothermal titration calorimetry, activity measurement and fluorescence spectroscopy to characterize the enzymatic reaction and determine the kinetical parameters.

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P24

ACQUISITION OF EXOGENOUS HAEM IS ESSENTIAL FOR TICK REPRODUCTION

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Haem and iron homeostasis in most eukaryotic cells is based on a balancing flux between the opposing pathways of haem synthesis in a multi-enzymatic pathway and haem degradation mediated by haem oxygenase (HO). In our work, we show that haem and iron metabolism in ticks depart from its canonical functioning described for other eukaryotic organisms [1].

We showed, by thorough mining in available genome and transcriptome databases, that ticks possess an incomplete haem biosynthesic pathway. Our experiments confirmed that ticks do not synthesise haem *de novo.* Instead, ticks acquire haem from host haemoglobin and recycle it as a prosthetic group needed for their endogenous haemoproteins. Reduced levels of dietary haem/haemoglobin led to aborted larvae hatching suggesting a critical involvement of host haem in the embryogenesis of ticks. As serum supplementation with haemoglobin or myoglobin, rather than equimolar supplementation with haemin, led to higher levels of haem deposits in tick eggs, we speculate that ticks express a specific receptor with affinity towards haemo-/myo-globin structure in the tick intestine. We further demonstrated that ticks, as well as other mites, lack the gene encoding HO. Loss of HO seems to be an ancestral trait in evolution of mites, followed by loss of haem biosynthesis in the origin of ticks. We have experimentally shown that ticks, indeed, do not acquire iron from host haem/haemoglobin but rather from a host transferrin, a major non-haem iron transporter protein found in mammalian blood. The mode of internalisation of host transferrin in the tick intestine is not clear as a gene orthologue of *bona fide* transferrin receptor was not found in the tick genome.

Tick midgut RNA-seq analyses of blood- and serum-fed ticks revealed a clear up-regulation of a transcript encoding a delta class Glutathione S-transferase (GST) in blood-fed group. Further analyses confirmed haemin-responsive expression of *gst*. Activity assays of His-tagged GST with model substrate 1-chloro-2,4-dinitrobenzene showed an inhibitiry effect of haemin but not of "empty" protoporphyrin IX on the enzymatic activity suggesting a potential binding of haemin to the recombinant GST. However, affinity of the non-his tagged recombinant GST to haem-related molecules still needs to be confirmed.

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P25

BROMODOMAINS AND CANCER EPIGENETICS: CONSEQUENCES OF NATURALLY OCCURRING MISSENSE MUTATIONS ON THEIR STRUCTURE AND FUNCTION

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Bromodomains (BRDs) are the only known small protein interaction modules that selectively targets -N-acetylation of lysines [1]. Lysine acetylation is one of the most frequently occurring post-translational modifications, so it is an important epigenetic mark regulating gene transcription and chromatin structure. Deregulation of acetylation levels has been associated with the development of many diseases and enzymes regulating acetylation have emerged as interesting targets for drug discovery. Recent studies revealed that BRDs are highly druggable protein interaction domains resulting in the development of a large number of bromodomain inhibitors [2]. In particular, inhibitors that specifically target the BET (bromo and extra terminal) proteins selectively interfered with gene expression that mediated cellular growth and evasion of apoptosis in cancer. Hence, BET inhibitors received a lot of attention in the oncology field resulting in the rapid translation into clinical studies.

BET proteins (BRD2, BRD3, BRD4 and BRDT) belong to the subfamily II of BRDs, sharing a common architecture comprising two N-terminal BRDs, with high level of sequence conservation, as well as an extra-terminal (ET) domain and a more divergent C-terminal recruitment domain. In our study we chose from COSMIC data base

(http://cancer.sanger.ac.uk/cosmic) some natural variants of domain 1 of BRD2 identified in cancer as nonsynonymous single nucleotide polymorphisms (nsSNPs), single nucleotide variations occurring in the coding region and leading to a polypeptide sequence with amino acid substitutions [3]. These variants have been expressed and purified as soluble recombinant proteins to investigate the effects of the amino acid substitutions on BRDs structure and stability and on the interactions with inhibitors, by means of CD and fluorescence spectroscopy and isothermal titration calorimetry.

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P26

ESTABLISHMENT OF INNOVATIVE, VASCULAR EQUIVALENTS FOR THE DEVELOPMENT OF DETECTION MODULES FOR HIGH-THROUGHPUT SCREENING AND FOR THE DEVELOPMENT OF ANTI-INFLAMMATORY PEPTIDES

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Tumor necrosis factor (TNF) alpha is a complex molecule, which is associated with many pathological processes. Especially the effect to the vascular endothelial is important because TNF is not only suspected to be involved in chronic inflammation but also appears to be responsible for a range of other cardiovascular diseases [1]. TNF is already used in tumor therapy by increasing the permeability of the tumor endothelia for cancer therapeutics [2].

We produced recombinant TNFReceptor2 in *P.pastoris* and used the soluble TNFR2 as a target for Phage Display screening. Phages are virus that are able to infect and replicate within a bacterium, a gene encoding a protein of interest is inserted into a phage gene, causing the phage to present the protein on its outside while containing the gene for the protein on its inside. These displaying phages can then be screened against other proteins, peptides or DNA sequences, in order to detect interaction between the displayed protein and those other molecules. We established the Phage Display Technology to isolate high affinity peptides, which binds to the central mediators (TNF and TNFR2) of inflammation processes [3].

Therefore, the recombinant protein was immobilized on nickel-agarose microplates with an N-terminal polyhistidine tag and the binding phage particles were eluted with a high excess of TNF alpha (competitively) to reach a highest possible specificity for TNF-alpha peptide ligands. The phage particles were amplified after three rounds “Biopanning” and analyzed for their binding specificity and strength by means of ELISA (Enzyme Linked Immunosorbent Assay) against TNF alpha. Promising candidates were also tested in a parallel-established Co-culture system for transendothelial migration, which can be also used for high-throughput screening. We detected the release of pro-inflammatory mediators (cytokines) as well as extravasation of lymphocytes through an endothelial monolayer and we analyzed to what extend the phage peptides can inhibit this processes.

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P27

ENZYMATIC ACTIVITY OF BGL2 IS NECESSARY FOR FORMATION OF TRIS-INEXTRACTABLE POOL OF BGL2 IN YEAST CELL WALL

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Cell wall of yeast and other ascomycetes mainly consists of glucan, which is synthesized as a linear polymer and then is modified by branching enzymes localized on the cell surface [1]. Domain architecture of most fungal GH17-family branching glucanases have features responsible for strong cell surface anchoring: TM-domain, GPI-anchoring signal or alkali sensitive linkage associated propeptide. Highly conserved Bgl2/Bgt1-orthogroup GH17-family glucanases consist of sole (/)8 TIM-barrel catalytic domain in mature form. Bgl2 from *Saccharomyces cerevisiae* strongly but non-covalently incorporates into yeast cell wall and demonstrates amyloid like properties [2], but partially can be extracted into 10mM Tis pH 9.0 [3]. Scw4 protein in yeast cell wall is similarly presented in two pools - non-covalently bound and covalently bound via alkali sensitive linkage forms [1]. Scw4 and other two yeast GH17-family glucanases have shortened version of GH17 catalytic domain when compared to Bgl2 [4], lacking C-terminal region corresponding to 8 of TIM-barrel [5].

Deletion of C-terminal 10 amino acids of Bgl2p in *S. cerevisiae* via site-directed mutagenesis resulted in protein instability and loss of SDS-treatment resistance. Among this 10 amino acids we identified that C310 is very conservative. C310A mutation led to mild sensitivity to SDS-treatment and decrease of Tris-inextractable pool percentage of cell wall Bgl2 (~5% of total cell wall Bgl2) when compared with wild type (~30%). C310 corresponds to cystein of GH17-family glucanase Bgt17A of *Rhizomucor meihei* that forms disulfide bond [5]. We suggest that disulfide bond is necessary for enzymatic activity via conformational stabilization of 8- 8 loop of catalytic domain, which contain 4 amino acids participating in substrate-binding as inferred from structure of *R. meihei* homolog [5]. E233A mutation of catalytic nucleophile in active site led to similar decrease in of Tris-inextractable pool of Bgl2 (~5%), supporting our suggestion that enzymatic activity in necessary for formation of Tris-inextractable pool of Bgl2p in cell wall.

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P28

CHARACTERIZATION OF THE PRDM9 ZINC-FINGER ARRAY BINDING AND MULTIMER FORMATION

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PRDM9 (PR-domain containing 9) has been identified as a meiosis specific protein that plays a major role in determining the location of meiotic recombination hotspots, but so far the binding characteristics of PRDM9 have still been enigmatic . PRDM9 is an epigenetic modifier which binds DNA via its long zinc-finger (ZnF) array and directs double strand breaks necessary for the initiation of recombination in its close vicinity . Motifs recognized by the ZnF array of PRDM9 are enriched at the center of the hotspots, yet these motifs are neither necessary nor sufficient to determine the binding , and it is still unclear what factors drive the binding affinity and specificity of the ZnF array in-vivo.

For this purpose we characterized the binding specificity of PRDM9 (murine CAST allele with 11 zinc-fingers) to a target recombination hotspot, *Hlx1*, in-vitro, using Electrophoretic Mobility Shift Assays. By consecutively replacing the specific target site by 5-nucleotide steps with a random DNA sequence, we observed that a minimal number of 15 nucleotides confer binding specificity, located in the middle or at the 5' end of the sequence, whereas, 15 nucleotides at the 3' end still confer binding, but at a much lower affinity. This is consistent with binding assays assessing the effect of single nucleotide changes and with the observation that motif enrichments at hotspots are much shorter than the minimal binding site , fitting the paradoxical observation that PRDM9 is highly specific and permissive at the same time. Furthermore we investigated the binding affinity of PRDM9 to its specific target DNA using gel shift assays as well as a more quantitative method, the switchSENSE technology from Dynamic Biosensors, to measure binding on and off kinetics result-

ing in a specific dissociation constant in the nM range. In order to assess whether PRDM9 binds as a multimer, we also used EMSAs. Therefore, we designed DNA sequences of different lengths containing one or two specific target sites for PRDM9 and determined the molecular weight of these protein-DNA complexes by comparing their migration distances on a native polyacrylamide gel. We tested different PRDM9 constructs missing distinct domains to also assess what regions of PRDM9 induce multimerization. In these experiments we demonstrated that PRDM9 forms functional multimeric complexes of at least two or more monomer units that are mediated within the C-terminal Zinc-Finger domain.

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P29

GROWTH HORMONE INDUCED IMMUNE RESPONSE AND ITS LIGAND BINDING INTERACTION

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The bovine growth hormone (bGH) is a 191 amino acid long endogenous protein. There is a recombinant exogenous form on the market (rbGH) which differs only one amino-acid from the endogenous form. The rbGH is administered biweekly to enhance milk production in dairy cows. This use is approved in several countries, for instance the United States, but is prohibited in the European Union and therefore requires detection of its use. After subcutaneous administration, the rbGH is excreted to the bloodstream of the cow in very low amounts. Research therefore focussed on biomarker profiling, which is an adequate approach to discriminate between treated and untreated animals [1]. Evaluation of the biomarkers showed the importance of an endogenous response, presumably induced antibodies against rbGH [2]. Therefore, future focus should be on in-depth characterization of the rbGH induced immune response and its ligand binding interaction system with the rbGH. With this focus, knowledge needs to be gained about the binding capacity of the rbGH induced antibodies, but also knowledge about the epitopes to which the growth hormone induced antibodies bind. Preliminary results of the epitopes recognized by the rbGH induced antibodies will be presented. But also the future focus, using the techniques offered in the course will be looked into. To focus on the fundamental understanding of the binding interaction system of rbGH with the rbGH induced antibodies to support the final aim; The design of improved on-site and laboratory-based detection systems for the determination of the presence of rbGH.

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P30

A NOVEL, MODIFIED ASYMMETRIC PCR AIDS THE APTAMER SELECTION PROCEDURE

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Aptamers are oligonucleotides that can bind to their targets with great selectivity and specificity similar to antibodies. In the last decades, the in vitro selection of aptamers from random DNA library was improved with several different methods. However, the efficient amplification and conversion of the dsDNA to ssDNA between the selection steps is not fully solved, yet. The amplification of the by-products by PCR and the loss of sequences during production of ssDNA hinders the selection.

We aimed at developing a new, modified asymmetric PCR to circumvent this shortcoming of aptamer generation. The key of our method is the addition of a 3' terminal blocked reverse primer to the PCR mixture. On one hand, the modification of reverse primer blocks the synthesis of the complementary strand thus directly provides ssDNA for the next selection cycle. On the other hand, this approach dramatically decreases the nonspecific annealing and consequently, the formation of PCR by-products.

We confirmed our findings by analysing the PCR products by acrylamide gel electrophoresis. According to the results of this analysis, the novel approach worked equally efficiently with using unique oligonucleotide and complex ssDNA library as template of the reaction.

We also studied whether the modified asymmetric PCR evades dsDNA to ssDNA conversion step of aptamer selection. To this end, we applied our previously published Alphascreen-based method by amplifying virus protein selective aptamer completed DNA library. The obtained data corroborated our hypothesis, enrichment of DNA library can be analysed without conversion of dsDNA.

Currently, we studied the effect of asymmetric PCR on the sequence diversity of ssDNA library by Next-Generation Sequencing.

P31

INVESTIGATING THE ROLE OF A DISORDERED REGION OF DPRE1 IN COMPOUND INTERACTION

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New anti-tubercular agents are needed for the treatment of tuberculosis as we enter an era of increasingly inadequate treatments for this infectious disease. The introduction of new compounds can be either alternatives for or the replacement of old drugs, thereby tackling the problem of multi-drug resistance.

Several new chemical entities have been discovered through the use of phenotypic screens, that were subsequently shown to inhibit DprE1. It is an essential enzyme which supplies arabinofuranose, a building block of the cell wall components arabinogalactan and lipoarabinomannan, and is highly druggable.

As a target to multiple small molecule inhibitors, it is crucial to understand how they interact with DprE1. To overcome a limitation of enzyme-ligand co-crystallization in my studies I have investigated the role of a disordered region of DprE1 thought to cover the active site in the substrate-bound state. Through the use of single amino-acid substitutions I have explored the effect specific mutations make in terms of ligand binding and enzymatic activity.

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P32

ELUCIDATION OF MOLECULAR MECHANISMS OF E3 UBIQUITIN LIGASE - E2 UBIQUITIN-CONJUGATING ENZYME PAIRINGS

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Ubiquitination, which is a covalent attachment of highly conserved 76 amino acid protein ubiquitin (Ub), is involved in the regulation of most cellular processes. Best known for its role in the degradation of proteins by the 26S proteasome, it can also modulate a plethora of non-proteolytic function, including vesicle trafficking, DNA repair, and importantly - immunity [1]. In essence, this reversible process is catalyzed by sequential action of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes.

E3s, which interact with both the E2 and the target and are the specificity determinants of the ubiquitination process [2], have been intensively studied with regard to their function in response to pathogens. In particular, a group of closely-related Plant U-Box (PUB) E3s, namely PUB22, PUB23, and PUB24, have been implicated in negative regulation of early immune signaling mediated by pattern recognition receptors [3]. Since E2s largely determine the Lys residue within Ub used to link the moieties in a chain and thus the fate of the modified target [4], it is essential to analyze the function of E3 ligases within the context of its interacting E2.

The Arabidopsis genome encodes 37 E2 enzymes, whereas more than 1400 E3s are predicted, indicating the presence of common features among certain subsets of E2s that determine specificity to a defined group of E3s. Protein-protein interaction studies involving bimolecular fluorescence-complementation assay supported by site-directed mutagenesis, *in vitro* protein-protein interaction studies and ubiquitination assays as well as information-driven molecular modeling will give insight into the molecular E2 – E3 networks that mediate ubiquitination.

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P33

STRUCTURAL AND BINDING PROPERTIES OF DNA RESPONSE ELEMENTS BOUND TO P53 PROTEINS AND THE ROLE OF SPACER SEQUENCES IN P53 DNA INTERACTIONS

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The tumor suppressor protein p53 binds sequence-specifically to defined DNA targets in the genome. The consensus DNA response element (RE) consists of two decameric half-sites (HS) with the general form RRRCWWGYYY (R = A, G; W = A, T; Y = C, T), separated by a variable number of base pairs (bp)(1). The binding of p53 to its RE is highly cooperative and relies on the recognition of DNA sequences by the core domain and on the tetramerization of the protein, which is heavily mediated by DNA (2, 3). Overall about 50% of all validated functional p53 REs have spacers between the half sites. Yet, functional binding i.e. binding that results in transactivation is more stringent and occurs from REs having either 0 or 1 bp spacer.

We have previously shown that DNA shape and flexibility can change significantly among different p53 REs (3). Here we use cyclization kinetics of DNA mini-circles in solution to investigate the static and dynamic properties of p53 response elements in their free and p53-bound states. We show that p53 does not bend its consensus binding sites when the two half-sites are either abutting or separated by long spacers, however there is an increase in the torsional flexibilities of many REs, upon p53 binding. Systematic binding experiments of consensus sites containing

spacer sequences show that the binding affinity is modulated by the relative orientation of the two half-sites. Binding sites with spacer sequences from 10 base pairs onwards show that p53 can bind its sites in two modes: sequence specifically and hemi-sequence specifically. The binding affinities of the two modes are similar, however, the hemi-specific complex competes better with genomic non-specific DNA in comparison to the specific complex. These findings further expand the "universe" of DNA binding sites of this multi-functional protein.

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P34

STRUCTURAL AND FUNCTIONAL STUDIES OF A COLD-REGULATED PAK-LIKE KINASE IN *TRYPANOSOMA BRUCEI*

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The main focus of my thesis is the structural characterization of a PKA-like kinase in the parasite *Trypanosoma brucei.* From yeast to humans PKA kinase is known to play an essential role in the transduction of cellular signal controlling several functions in the cell, including regulation of glycogen, sugar and lipid metabolism [1]. PKA is ubiquitously known for being activated by the second messenger cyclic adenosine monophosphate (cAMP) that is generated by adenylyl cyclases in response to an external signal. In *Trypanosoma brucei* this kinase was found to be activated via "cold shock" upon a temperature drop from 37şC to 20şC reflecting the temperature shift the parasite faces when transmitted from the human blood to the midgut of the Tsetse fly [2].

In this project we aim to investigate the binding properties of this second messenger to the regulatory subunit of the PKAR in *Trypanosoma brucei*. Recently we obtained the crystal structure of the regulatory subunit of this PKA from *T. brucei* as well as from the related parasite *T.cruzi*, the causative agent of Chagas disease. We aim at understanding the physiological activation mechanism of this kinase, specifically the interplay between temperature and ligand binding. To this end a number of site-directed mutants will be subjected to binding assays under different temperatures to test hypotheses relative to cold shock activation.

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P35

CHARACTERIZATION OF INTERACTION BETWEEN HDAC6 AND TUBULIN DIMERS/MICROTUBULES

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In the cell, tubulin undergoes post-translational modifications that create functionally distinct microtubules and mark them for specialized functions [1]. One of these is acetylation at Lys-40 of -tubulin that is controlled by tubulin acetyltransferase and histone deacetylase 6 (HDAC6), respectively. HDAC6 is a unique class IIb mammalian HDAC that localizes predominantly to the cytoplasm and exerts the deacetylase enzymatic activity mainly on nonhistone substrates, such as tubulin and survivin [2].

Neither the atomic structure of HDAC6 nor details of interactions governing substrate recognition by HDAC6 is known at present. Mapping interaction between HDAC6 and its physiological substrates, especially tubulin, would provide critical insight into functioning of the enzyme. The main goal of the project is to describe the molecular basis of the substrate recognition by HDAC6 and to find out substrate preference. While studying HDAC6/tubulin interactions by single molecule methods using TIRF microscopy we confirmed HDAC6 binding on microtubules. So deter-

mining of the affinity of different HDAC6 variants for tubulin dimers/microtubules using a combination of biochemical and biophysical experimental methods to map HDAC6/tubulin interface by employing complementary qualitative and quantitative methods as SPR and thermophoresis will help to understand this interaction.

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P36

MODULATION OF FC RIIIA IN THE TREATMENT OF AUTOIMMUNE DISEASE

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Rheumatoid arthritis (RA) is an autoimmune disease affecting ~1% of people worldwide that rapidly leads to joint damage if left untreated. The presence of autoantibodies is a major poor prognostic factor in RA. Approximately 80% of patients are seropositive for autoantibodies, whose main receptors are Fc Receptors (Fc R), which bind the Fc portion of IgG and are responsible for transducing downstream effector functions such as phagocytosis, cytokine release and antibody-dependent cell-mediated cytotoxicity (ADCC).[1, 2]

Therapeutic modulation of the interaction between IgG-containing autoantibody immune complexes and Fc Rs is highly desirable. In particular, Fc RIIIa is known to mediate pro-inflammatory signalling upon binding IgG-containing autoantibody immune complexes [3], and we have generated artificial binding proteins (Adhirons [4]) specific for Fc RIIIa which demonstrate functional IgG blocking.

Co-crystallisation of Fc RIIIa with Adhiron illustrates an allosteric effect on the receptor, responsible for disrupting the IgG-Fc R interaction through steric restraint, but does not reveal the structural basis of selectivity over the highly similar Fc RIIIb, which shares 96% sequence homology with Fc RIIIa. This allosteric site is potentially a novel therapeutic site for disrupting the IgG-Fc R interaction, and dissection of the Fc R-Adhiron interaction and understanding the basis of the Adhiron selectivity for Fc RIIIa is of great importance. SPR studies to evaluate affinity have suggested unexpectedly low affinity of Adhiron for Fc RIIIa. Initial studies using MST have provided a more realistic affinity, however more robust biophysical data are required to validate our system fully.

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PLASMODIUM REICHENOWI EBA-140 MEROZOITE LIGAND RECOGNIZES THE HOMOLOG OF GLYCOPHORIN D ON CHIMPANZEE ERYTHROCYTES

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Plasmodium reichenowi, the parasite which infects chimpanzees, is morphologically identical and genetically similar to *P. falciparum*, a human malaria parasite responsible for malignant malaria. Genomes comparison of *P. falciparum* and *P. reichenowi* reveals major differences in genes coding for proteins engaged in erythrocytes invasion[1].

Erythrocyte binding ligand 140 (EBA-140) is a member of the *P. falciparum* erythrocyte binding antigens (EBA) family[2]. The amino acid sequence of *P. reichenowi* EBA-140 ligand is 81% identical with *P. falciparum* binding region (Region II), which is responsible for ligand-erythrocyte receptor interaction during merozoite invasion[3]. Until now the receptor for *P. reichenowi* EBA-140 ligand on chimpanzee erythrocytes was not identified.

In order to evaluate the molecular basis of the erythrocyte recognition by *P. reichenowi* EBA-140 ligand, we expressed its binding region (Region II) using baculovirus expression vector system in HighFive insect cells, similar as homologous *P. falciparum* recombinant Region II[4]. The protein secreted into the medium was simply purified in one step process by NiNTA affinity chromatography. Binding of EBA-140 Region II to erythrocytes was examined by flow cytometry, surface plasmon resonance and immunoblotting using native, neuraminidase-, trypsin- or chymotrypsin- treated chimpanzee red blood cells.

The recombinant baculovirus-expressed EBA-140 Region II binds to normal chimpanzee erythrocytes in a dose dependent manner. It binds trypsin-treated chimpanzee erythrocytes, but its binding to erythrocytes treated with neuraminidase or chymotrypsin was significantly decreased. These results indicate that the recombinant protein is functional and its interaction with chimpanzee erythrocytes is specific and sialic acid-dependent. Moreover, the observed enzymatic profile of Region II binding pointed at glycophorin D homolog, as a putative receptor for *P. reichenowi* ligand EBA-140 on chimpanzee erythrocytes.

The soluble, functional recombinant *P. reichenowi* EBA-140 binding region was obtained for the first time. We have characterized the molecular basis of its specificity to chimpanzee erythrocytes. Further ligand-binding studies will be performed to identify the receptor site on glycophorin D responsible for this interaction, especially with respect to sialic acid molecule.

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