B1–Proteases as Molecular Targets of Drug Development

B1-001
DPP-IV structure and inhibitor design

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The incretin hormones GLP-1 and GIP are released from the gut during meals, and serve as enhancers of glucose stimulated insulin release from the beta cells. Furthermore, GLP-1 also stimulates beta cell growth and insulin biosynthesis, inhibits glucagon secretion, reduces free fatty acids and delays gastric emptying. GLP-1 has therefore been suggested as a potentially new treatment for type 2 diabetes. However, GLP-1 is very rapidly degraded in the bloodstream by the enzyme dipeptidyl peptidase IV (DPP-IV; EC 3.4.14.5). A very promising approach to harvest the beneficial effect of GLP-1 in the treatment of diabetes is to inhibit the DPP-IV enzyme, thereby enhancing the levels of endogenously intact circulating GLP-1. The three dimensional structure of human DPP-IV in complex with various inhibitors

creates a better understanding of the specificity and selectivity of this enzyme and allows for further exploration and design of new therapeutic inhibitors. The majority of the currently known DPP-IV inhibitors consist of an alpha amino acid pyrrolidine core, to which substituents have been added to optimize affinity, potency, enzyme selectivity, oral bioavailability, and duration of action. Various compound series and their SAR relative to alpha amino acids will be presented.

B1-002
MEROPS: the peptidase database

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Peptidases (also known proteases or proteinases) and their inhibitors are of major importance to human health. The *MEROPS* database is a comprehensive information resource on these proteins freely available to all. Central to the database is the hierarchical classification system first introduced by Rawlings & Barrett (1993), which is now almost universally accepted. Peptidases are classified according to biochemical characterization, sequence homology and structural similarity. For each peptidase a region known as the peptidase unit is defined, which encompasses the structural domains and residues important for activity. Each peptidase is given a unique MEROPS identifier, peptidases with homologous peptidase unit sequences are grouped into a family, and peptidase units with similar structural folds are grouped into a clan (which can contain one or many families). A similar classification was developed for the protein inhibitors of peptidases in 2004. Records can be accessed through the indexes, which list peptidases by MEROPS identifier, alphabetically by name (including numerous synonyms), or by source organism name (both scientific and common). The database provides a gateway to the extensive literature on peptidases, and the current release of the database includes over 20 000 references. There are annotated alignments at clan, family and peptidase levels showing peptidase units, active site residues and other structural features, and evolutionary trees for each family and peptidase. There are comprehensive searches of EST alignments showing alternatively spliced variants and SNPs that change the protein sequence.

B1-003
Structure and function relationship of memapsin 2 (beta-secretase)

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Memapsin 2 (β-secretase, BACE1) is the membrane-anchored aspartic protease that initiates the cleavage of β-amyloid precursor protein (APP) leading to the production of amyloid-β (Aβ), a major factor in the pathogenesis of Alzheimer's disease (AD). Since memapsin 2 is a major target for the development of inhibitor drugs for the treatment of AD, its structure and physiological functions are topics of intense research interest currently. Here we discuss the structural features of memapsin 2 and how do they contribute to the activity and inhibition of the protease. Structural and kinetic evidence support the presence of 11 subsites for substrate or inhibitor binding in the active-site cleft of memapsin 2. Subsites P3 to P2' are most useful in the design of transition-state analogue inhibitors. Recent data indicated that subsites P7, P6 and P5 have strong influence of hydrolytic rate or inhibition potency. These subsites are, however, too far from the transition-state isostere for the design of drug-like transition-state inhibitors but can be utilized for the design of non-transition-state inhibitors that compete for substrate binding. Besides carrying out proteolytic activity, the ectodomain of memapsin 2 also interacts with APP leading to the endocytosis of both proteins into the endosomes where APP is hydrolyzed by memapsin 2 to produce Aβ. A phosphorylated motif in the cytosolic domain of memapsin 2 is responsible for the recognition of GGA proteins as part of the recycling mechanism that transports memapsin 2 from endosomes to trans-Golgi then back to cell surface. These interactions may also be considered for the design of small-molecular compounds that interfere with memapsin 2 trafficking and thus reduce the production of Aβ.

B1-004
Identification of human carnosinase – a brain-specific metalloprotease

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Metalloproteases form a large and diverse family of proteases and are molecular targets that represent an opportunity for therapeutic intervention. In particular, the development of potent inhibitors has made progress for the family of matrix metalloproteases (MMP). The sequencing of the human genome revealed that a significant percentage of the drugable genome is represented by proteases, many of them still with unknown function. In this presentation, data will be presented on the deorphanization of two previously unknown genes by means of bioinformatics and classical biochemistry. This work led to the identification of human carnosinase, a dipeptidase specifically expressed in the human brain and an ubiquitously expressed close homologue, characterized to be a non-specific dipeptidase.

B1-005
Stimulating serpins with synthetic tailor-made oligosaccharides: a new generation of antithrombotics

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We will discuss our research on synthetic oligosaccharides able to selectively activate the inhibitory activity of antithrombin towards various serine proteinases. We first synthesized pentasaccharides closely related to the antithrombin binding domain of heparin [1] (the active site), as well as analogues displaying different pharmacokinetic profiles. Selective inhibitors of coagulation factor Xa were thus obtained that represent a new class of antithrombotic [2] drugs currently being evaluated worldwide. We then designed larger oligosaccharides [3] that inhibit both factor Xa and thrombin in the presence of antithrombin. They are devoid of undesired non-specific interactions with blood proteins, particularly with platelet factor 4. Clinical trials are ongoing to prove the therapeutic benefits of this new type of coagulation inhibitors.

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B1-006
Slow tight binding inhibitors in drug discovery: in the case of DPPIV and elastase inhibitors

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Enzyme are extremely potent causing significant inhibition at very low concentrations that may be comparable to the concentration of the target enzyme. When this inhibition is studied *in vitro*, complexities arise because the concentration of the inhibitor is so low that it is altered significantly as a result of combination with the enzyme. This situation is referred to as tight-binding inhibition. Partly as a result of their low concentrations, tight-binding inhibitors often show slow-binding characteristics. Unlike conventional inhibitors that act almost instantaneously (or at least within the ms time scale), slow-binding inhibitors may take several seconds, minutes or even hours for their effect to be fully exhibited. This association between slow-binding and tight-binding is relatively common and slow tight-binding inhibitors are extremely potent and specific. Proteolytic enzymes are involved in a multitude of important physiological processes. Their intrinsic properties and activities are in the focus of wide-ranging research and they have a valuable role in experimental and therapeutic purposes. Serine proteases are attractive targets for the design of enzyme inhibitors since they are involved in the etiology of several diseases. Within the class of serine proteases, human leukocyte elastase (HLE) is one of the most destructive enzymes in the body. The enzyme dipeptidyl peptidase IV (DPPIV) is a serine exopeptidase that cleaves Xaa-Pro dipeptides from the N-terminus of oligo-and polypeptides. Inhibitors of DPP IV are of increasing interest to pharmaceutical industry alike, as they may become established as the next member of the oral antidiabetic class of therapeutic agents. Objective of our work was to develop reversible, slow, tight-binding inhibitors against these serine proteases. SSR69071 is a potent inhibitor of HLE, the inhibition constant (K_i) and the constant for inactivation process (k_{on}) being 0.0168 ± 0.0014 nM. This inhibitor is reversible, slow, tight-binding inhibitor with k_{on} = 0.183 ± 0.013 10^6/ms, and k_{off} = 3.11 ± 0.37 10^{-6}/s. SSR69071 inhibits the solubilization of elastin by HLE with 13 nM of IC50 value. This inhibitor is one of the most effective inhibitor of a serine proteinase yet described. SSR162369 is a potent, competitive and slow tight binding type inhibitor of the human dipeptidyl peptidase-IV enzyme (K_i = 2 nM, $T½$ = 8 h). On the basis of kinetic properties, SSR162369 forms stable enzyme-inhibitor complex. These slow tight-binding inhibitors have unique inhibitory properties, they are extremely active, and selective, form stable enzyme–inhibitor complex, therefore they have long-lasting effect. Their oral activity and long lasting *in vivo* biological potency agreed very well with stable enzyme–inhibitor complex. The advantages in drug discovery of slow tight-binding inhibitors are discussed in this presentation.

B1-007P
Enzyme inhibition trend analysis – a new method for drug design

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Many of the drugs that are currently in use or at different stages of development are enzyme inhibitors. Therefore, enzyme mechanism-based inhibitors could be developed into highly selective drugs. Our novel enzyme inhibition trend analysis method combines experimental enzyme kinetics data and high level quantum mechanical modeling of enzyme–inhibitor chemical interactions. The method utilizes the principal catalytic reaction scheme of the target enzyme and does not require its 3D structure (a ligand based approach). The method is valid for the prediction of the trend in binding affinity of inhibitors not only for the specific enzyme for which the QSAR model was optimized, but also for the whole enzyme family. The methodology would contribute significantly to overcoming the problem of fast mutational resistance developed by pathogens in response to pharmaceutical treatment. It can be used as a computational tool for expert analysis of various hypotheses about structure–activity relationships formulated for the design of new inhibitors.

B1-008P
Dramatical role of ligand length in the effectivity of angiotensin-converting enzyme inhibition

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Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a key enzyme for blood pressure control and water-electrolyte homeostasis. A large number of highly potent and specific ACE inhibitors are used as oral drugs in the treatment of hypertension and congestive heart failure. Somatic ACE consists of two homologous domains (N- and C-) within single polypeptide chain, each one containing a catalytic site. The two catalytic sites within somatic ACE molecule were long considered to function independently. However, recent investigations indicate the existence of negative cooperativity between ACE active sites. We studied the properties of bovine ACE active centers by use of separate ACE N-domain (N-ACE) obtained by limited proteolysis of parent somatic enzyme and testicular ACE, which represents C-domain. These results were compared with the data obtained for full-length somatic ACE from bovine lungs. The results obtained demonstrate strongly dependent mechanism of action of ACE active centers in the reaction of the hydrolysis of tripeptide substrates. However, the hydrolysis of decapeptide angiotensin I proceeds independently on N- and C-domains. The mechanism of inhibition of ACE activity is also dependent on the length of the inhibitor: (i) random binding of the "short" inhibitor molecule (such as captopril, lisinopril) to one of the active sites dramatically decreases binding of another inhibitor molecule to the second site; (ii) "long" nonapeptide teprotid binds to both active sites without any difficulties. Since the main physiological ACE substrates in the organism are "long" peptides angiotensin I and bradykinin, the development of new class of inhibitors with prolonged structure would be beneficial for abolishing of ACE activity.

B1-009P
Synthetic peptide studies on severe acute respiratory syndrome coronavirus (SARS-CoV)

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The SARS-CoV swept the world in early 2003. SARS viral genome encoded functional polypeptides are released from the

extensive proteolytic processing of the replicase polyproteins, pp1a (486 kDa) and pp1ab (790 kDa), by the SARS-CoV 3C-like protease ($3CL^{pro}$). Besides, the structural spike protein of SARS-CoV contains two heptad repeat regions (HR1 and HR2) that form coiled-coil structures, which play an important role in mediating the membrane fusion process. In this study, we focused on both $3CL^{pro}$ and the HR regions of SARS. Previous studies demonstrated that the coronavirus $3CL^{pro}$ cleaves the replicase polyproteins at no <11 conserved cleavage sites, preferentially at the LQ sequence. The reported crystal structure of SARS-CoV $3CL^{pro}$ provides insights into the rational design of anti-SARS drugs. In order to understand the molecular basis of the enzyme-substrate binding mechanism, we employ the synthetic peptide and mass spectrometry-based approaches to investigate the significance of selected amino acid residues that are flanking both sides of the SARS-CoV $3CL^{pro}$ cleavage site. In addition, previous studies indicated that the relatively deep hydrophobic coiled coil grooves on the surface of SARS-CoV spike protein heptad repeat regions (HR1 and HR2) may be a good target site for the design of viral fusion inhibitors. We have designed and synthesized five truncated peptide analogs derived from HR1 and HR2 peptides based on both bioinformatics and structural analysis. The biological activities of these truncated analogs will be studied using circular dichroism spectroscopy, multidimensional chromatography, protein cross-linking and mass spectrometry-based approach. The above investigation will definitely broaden our knowledge on the SARS research and will reveal the feasibility of rational design of synthetic peptide-based drug in combating with SARS disease.

B1-010P
Effects of Seropharmacological traditional Chinese drug on proliferation of rat mesenchymal stem cell *in vitro*

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Objective: To observe the effect of Seropharmacological traditional Chinese drug on proliferation of rat mesenchymal stem cell (MSC) *in vitro*.
Methods: Mesenchymal stem cells were dissociated from rat bone marrow and were marked by Brdu, and the expression of CD44. CD 54 and double label of Brdu and CD 44.The growth of rat mesenchymal stem cell under Seropharmacological traditional Chinese drug were observed by means of cell viability measurement (MTT)and morphological observation and Brdu,PCNA immunohistochemical methods.
Results: Seropharmacological traditional Chinese drug can promote the cell viability of MSC and the number of Brdu, PCNA positive cell in dose-dependant, there are significant difference in comparison with control groups.
Conclusion: Seropharmacological traditional Chinese drug had strong effect on enhancing proliferation of MSC
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B1-011P
Ras-transfection-associated invasion: involvement of matrix metalloproteinase(s) confirmed using a chicken embryo model and real time PCR

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During metastasis tumorogenic cells leave the primary tumour and intravasate into the blood/lymphatic system, exiting at a secondary site to establish a secondary tumour. Ras-transfection of a parental, non-invasive MCF-10A cell line, established from a patient suffering with benign fibrocystic disease, gave rise to an invasive derivative cell line (MCF-10A-NeoT) exhibiting the phenotype of a pre-malignant, invasive tumour. Invasion and metastasis are protease-assisted processes, proteases either being secreted by the tumour, or by the stromal cells under the influence of the tumour. Here we demonstrate the involvement of matrix metalloproteinase(s) in the invasion of the ras-transfected MCF-10A cell line. Tumour cells were inoculated onto the damaged surface of the upper chorioamniotic membrane (CAM) of a vasculated 9-day old chick embryo. The tumour cells were allowed to invade, and the number of invading cells quantified using real time PCR. Inhibitors specific for various proteases were applied to the upper CAM, to block invasion, and hence identify the proteinases involved. The number of tumour cells invading into the vascular system was established by sampling the lower CAM and quantifying the numbers of Alu sequences (present only in human cells) in the DNA, isolated from the embryonic tissue, using real-time PCR. Using this method, the key role of an MMP was demonstrated.

B1-012P
Matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 release from human neutrophils

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Matrix metalloproteases (MMPs) seem to be involved in neutrophil invasion, inflammation and tissue damage, processes potentially limited by tissue inhibitors of metalloproteinases (TIMP)-1. TIMP-1 is separately localized from target MMPs, and may potentially be differentially released, providing an opportunity for therapeutic intervention. Preliminary results indicate that both MMP-9 (the predominant MMP in neutrophils) and TIMP-1 are constitutively released in physiological saline and HEPES, but together with increasing extracellular calcium, a bi-phasic release pattern was observed. The broad

spectrum PTK inhibitor, genistein (100 μM) abolished the release of neutrophil MMP-9, in the presence and absence of extracellular calcium, and reduced the release of TIMP-1. Both PP2 (10 μM), a Src family PTK inhibitor, and piceatannol (30 μg/ml), a Syk family PTK inhibitor, reduced MMP-9 release substantially, indicating that multiple PTK families might be involved in MMP-9 release. Inhibition of either Syk or Src PTKs by piceatannol or PP2 did not appear to influence TIMP-1 release. Low levels of wortmannin (100 nM, inhibition of PI3K) abolished the release of MMP-9 in the absence of calcium, and reduced MMP-9 release in the presence of calcium. Investigations into the signaling pathways involved in TIMP-1 release are continuing. We conclude that MMP-9 release induced by extracellular calcium may be mediated through PI3K and multiple tyrosine kinases, including Src and Syk family PTKs. TIMP–1 granule release may also be mediated by tyrosine kinases, although Src and Syk family PTKs do not appear to be involved.

B1-013P
Thermodynamical and structural analysis of cruzain/cruzipain2 complexed with E-64 by molecular modeling and dynamics simulations

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Aim: New opportunities for structure-based design of anti parasite drugs have emerged from studies of a family of structurally homologous cysteine proteases, identified in several pathogenic parasites. The major cysteine protease from *Trypanosoma cruzi* (cruzipain/cruzain) is a member of a polymorphic multi-gene family, with a structural organization similar to papain-like mammalian lysosomal proteinases. Current studies indicate that cruzipain2, a cruzipain isoform, exhibits kinetic properties that are different from those of cruzipain.
Objectives: We present a new approach to estimate relative interaction affinities of the catalytic site of enzymes and inhibitors, using crystal structures as well as modeled structures and performing Molecular Dynamics (MD) simulations and thermodynamical calculations by Gibbs–Helmholtz equation. We have tested this methodology using the E-64 in complex with cruzain and cruzipain2 enzyme isoforms. MD simulations in explicit SPC water were performed for 2.0 ns at 295, 305, and 315 K. The energies of E64/cruzain and E64/cruzipain2 complexes were compared with the enzymes without inhibitor to obtain the differences in the total energy variations to form the complexes. With data of enthalpy vs. temperature, thermodynamical parameters were obtained to estimate the Gibbs free energy variation (ΔG^0) and the affinities of the inhibitor to enzymes.
Conclusion: Our thermodynamical analysis indicates that the E-64 in the catalytic site of these enzymes is energetically more favorable in cruzain compared to cruzipain2 at the range of temperature studied, showing a good accordance with experimental data. We verified that there is a drastic difference in the E-64 positions in a cleft catalytic of these enzymes.

B1-014P
An integrated platform for high throughput expression of human peptidases

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Peptidases represent one of the most relevant enzyme classes targeted by therapeutic intervention. To contribute to the assignment of a physiological role to genomic-derived peptidases and to make them more accessible for the drug discovery process, we have undertaken a program consisting of mRNA expression profiling, full-length recombinant expression in insect cells, purification and determination of the catalytic activity for the human proteolytic enzymes. A milestone in the process was the construction of a non-redundant comprehensive database for all human peptidases comprising 443 unique annotated entries, by assembling and filtering public domain information and in-house generated data. In order to get an informative picture on their expression profiling, a transcriptome database for 375 human peptidases was created using the microarray (Affymetrix™) and TaqMan® (Applied Biosystems) technologies. In parallel, we have set up the procedure for PCR amplification and cloning of the peptidase genes in 96 MTP format and we have already created a repository of 231 full-length human cDNAs encoding for peptidases. Besides, the conditions for miniaturized insect cell cultures have been established. Experimental trials have defined a validated, reliable and fully-automated robotic procedure for the purification of recombinantly expressed peptidases in 96 MTP format. In a pilot study using the high-throughput approach, 85% of the chosen reference hydrolases (14) were secreted into the insect cell medium. Of them, 66% have been proven to be catalytically active using fluorescent homogeneous assays in 384-well format compatible with the high-throughput screening criteria. The application of this procedure to genomic-predicted peptidases is discussed.

B1-015P
Comparison of putative glutamate racemases from *Bacillus* species

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Glutamate racemase catalyzes the interconversion between L- and D-glutamic acid and is the cell's source of D-glutamate, a key component in the synthesis of both the bacterial cell wall and the glutamyl capsule. *Bacillus subtilis* has two glutamate racemases in its genome, RacE and YrpC, while *B. cerus* and *B. anthracis* have two RacE genes, RacE1 and RacE1. Interestingly, RacE in *B. subtilis* is the isoform that is essential and has the greater catalytic efficiency, but both RacE1 and RacE2 have higher sequence homology to RacE, 70 and 79% respectively and share less homology with the YrpC isoform, both at 53%. We have cloned, overexpressed, purified, and are characterizing the kinetic and biophysical properties of the two putative glutamate racemases, RacE1 and RacE2 from *B. cereus* and *B. anthracis*, and will utilize kinetic and biophysical information to design inhibitors that may result in a novel antibiotic. Although these two isoforms share a high sequence similarity, their properties are unique. Kinetic data indicates a fivefold difference in catalytic efficiency of RacE2 compared to that of RacE1 in the L- to D- glutamate reaction. Also, the absence or presence of substrate has an effect

on the oligomerization state, details of which will be reported. Finally, our collaborators have demonstrated through genetic knock out experiments that only one of the RacE isoforms is essential for the growth of *B. anthracis*. We have crystallized the RacE2 isozyme and X-ray data have been collected to 2.3 Å. We are currently solving the structure via heavy-atom derivatives.

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B1-016P
Anti-inflammatory effects of methionine aminopeptidase 2 inhibition on human B lymphocytes

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Processing of N-terminal methionine is an essential post-translational modification in both prokaryotes and eukaryotes regulating the subcellular localization, stability and degradation of proteins. The cleavage of the initiator methionine is catalysed by a highly conserved family of metalloproteases, Methionine-aminopeptidase 1 and 2 (Met-AP2). Human Met-AP2 is the molecular target of fumagillin, a natural product with antiangiogenic properties, which covalently binds to His 231 in the catalytic site of Met-AP2. Although fumagillin has been observed to inhibit proliferation and to cause cell cycle arrest in endothelial cells, the mechanism of inhibition is still poorly understood. Recent studies describe high expression of Met-AP2 in germinal centre B lymphocytes. Here, we investigate the effect of the Met-AP2 inhibitor fumagillin on B lymphocyte proliferation and cell cycle progression and compare these results to those observed in HUVEC. In addition our work sheds light on the mechanistic aspects of Met-AP2 inhibition by fumagillin and its derivatives.

B1-017P
Effect of distal mutations on the molecular dynamics of the HIV-1 protease

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L10I and L90M are the most common distal mutations found in the protease gene of the drug resistant HIV-1 strains. These mutations do not confer resistance by themselves, however induce a large synergy effect when added to active site mutations. Understanding the impact of the L90M and L10I mutations on the HIV-1 protease resistance profile is still a challenge. Assuming that their contribution to the resistance profile could be mediated by conformational dynamics we have modeled L10I, L90M and L10I/L90M mutants of HIV-1 protease. These unbound mutated and wild type proteases were subjected to 10ns molecular dynamics simulations and compared using an Essential Dynamics (ED) analysis protocol. The first eigenvector of the native protease describes the flap openning motion. Following eigenvectors describe "the catalytic assisting motions" (CAM) of the protease that becomes dominant upon complex formation with a substrate (Piana S et al. *J Mol Biol* 2002; **319**(2): 567–583). Mutation of luecine to methionine residue at position 90 perturbs the protein packing at the dimerization domain. Such perturbations affect the dimerization domain motions which correlate with flap opening and the CAM. As result the first eigenvector corresponds to the rotational of the one subunit relative to another along axis connecting residues 60 and 60'. In other words L90M mutation mistunes essential motions of the enzyme while retaining its flexibility. This could be the cause of the reduced structural stability of the L90M mutant. In contrast, L10I mutation causes only redistribution of the correlated motions amplitude. The catalytic assisting motion becomes the most influential that results in stabilization of the closed conformation. In turn, the flap opening motions are reduced in L10I mutant. Essential dynamics of the double mutant L10I/L90M could be described in the following terms. A strong propagation of the CAM induced by L10I mutation is coupled with the altered conformational space caused by L90M mutation. As result the double mutant prefers CAM motions that are close to the native protease but also account for the perturbed packing within the dimerization domain. Results presented may help understanding HIV-1 protease resistance pathways and in developing more efficient inhibitors of known drug resistant mutants.

B1-018P
Glutamate carboxypeptidase II as a cancer marker and therapeutical target: two faces of an enzyme

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Glutamate carboxypeptidase II is a membrane-bound metallopeptidase expressed in a number of tissues such as jejunum, kidney, prostate and brain. The brain form of GCPII (also known as NAALADase) is expressed in astrocytes and cleaves N-acetylaspartyl glutamate, an abundant neurotransmitter, to yield free glutamate. GCPII thus represents an important target for the treatment of neuronal damage caused by excess glutamate. Animal model experiments suggest that specific inhibitors of GCPII could be useful for the treatment of several neuropathic conditions, such as brain stroke, chronic neuropathic pain or amyotrophic lateral sclerosis. In the same time, the enzyme is known as prostate-specific membrane antigen since it is upregulated in prostate cancer. It is used for the diagnosis and experimental therapy of prostate cancer using monoclonal antibodies and specific inhibitors. In order to analyze this important pharmaceutical target, we established an expression system based on *Drosophila* Schneider's cells. We have also cloned, expressed and characterized its human homolog GCPIII and homologous carboxypeptidases from pig and rat. Using specific monoclonal antibodies, we have been able to study the expression of GCPII in various healthy and malignant tissues. We analyzed the substrate specificity of the enzyme using peptide libraries and identified two novel peptide substrates. Availability of a recombinant protein enabled to introduce a simple fluorescent activity assay and test specific inhibitors. Furthermore, we have biochemically characterized the recombinant protein in terms of pharmacologic properties, oligomeric status, pH dependence and activity modulation by metal ions. We have shown that the glycosylation is indispensable for GCPII carboxypeptidase activity and analyzed the role of each specific N-glycosylation site for the GCPII activity and folding. Using site-directed mutagenesis, we are able to identify the domains sufficient and necessary for GCPII activity and also suggest structural explanation for the substrate specificity of the enzyme.

B1-019P
Doxycycline effect on metalloproteinases depends on the ECM environment

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Background: Metalloproteinases (MMP) are a family of proteinases with roles in epidermal wound healing. Periostat (Doxycycline hyclate) is the only MMP inhibitor on the US market. For more effective use of Periostat in wound healing, understanding its mechanism of action is important. Since extracellular matrix (ECM) regulate MMPs, we hypothesized that doxycycline hyclate-modulation of MMP expression would vary with specific ECMs.
Methods: Primary cultures of normal oral epithelial (NOE) cells from gingival biopsies were grown for 24 h in keratinocyte media supplemented with laminin and fibronectin, and then treated with doxycycline for an additional 24 h. Culture media were collected and expression of MMP9 was evaluated by zymography.
Results: Four experiments were performed. Analysis of variance showed: (i) a significant ECM-effect on the expression of pro-MMP 9 ($P < 0.05$), laminin having an inhibitory effect compared to fibronectin and media alone; (ii) a significant time effect $P < 0.05$), the expression of pro-MMP 9 being higher at day 2; (iii) a significant drug effect due to inhibition of pro-MMP 9 expression ($P < 0.05$) and (iv) a significant interaction between drug and ECM factors ($P < 0.05$). In the presence of media and fibronectin, doxycycline inhibited the expression of pro-MMP 9 by only 10 and 3% respectively. However, in the presence of laminin, doxycycline inhibited pro-MMP 9 by 50% suggesting an interaction between laminin and doxycycline effects.
Conclusion: The results of this study suggest that the doxycycline hyclate modulates the expression of pro-MMP 9 in NOE cells and this modulation depends on ECM environment.

B1-020P
Modeling, synthesis and application of H-bonding chemicals for cancer therapy to restore levels of oncosupressors by inhibiting protease activity of 20S proteasomes

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Dozens of chemicals feature inhibition of proteolytically important tyrosine residue of 20S proteasome by forming covalent bond to hydroxyl group that abolished its catalytic function. In contrary, the approach we utilize here is based on hydrogen and hydrophobic interactions reversibly inactivating all three sites of 20S complexes. We performed flexible docking studies of analogues of a natural product TMC-95A using 1JD2 crystal structure to describe the active site of protein and the position of the ligand. The search yielded several amide-like derivatives that have been screened for superimposition with TMC-95A. Few of them revealed similar orientation of propylene groups to the active site of 20S. Second screen was performed to reveal the chemicals with the strongest hydrogen-bonding of the ligand to the protein backbone of the receptor. This screen resulted in two chemicals that had strong H-contacts with Tyr21, Ser129 and, importantly, with proteolytically active Tyr1 residue. To access the validity of the predicted chemicals we undertook *in vitro* studies measuring the hydrolyses of fluorogenic substrate by the SDS activated 20S proteasome isolated from HeLa cells. We obtained more than 85% inhibition of 20S proteasome activity upon incubation the above chemicals (0.5 μg/ml) with proteasomes. We then demonstrated the effectiveness of the obtained chemicals to stabilize the level of oncosupressors, including p53 in benign (MCF10A) and highly metastatic (MDA231) cell lines. Treatment with these compounds greatly restored the level of p53 in cancer cells. Finally, we performed proliferation assay and proved that adding of this artificially synthesized chemicals to MDA231 cell line significantly reduced the level of proliferation, whereas MCF10A cells treated at similar conditions have not revealed any abnormal reduction of proliferation below control level. Thus, we report of a strategy to predict highly suitable proteasome inhibitors that act via inhibition of protease activity and may lead to creation of a new class of drugs for cancer therapy.

B1-021P
Localization and trafficking of prostate specific membrane antigen (PSMA) and its variant form PSḾ

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Glutamate carboxypeptidase II, also known as prostate specific membrane antigen (PSMA), is a transmembrane glycoprotein highly expressed in maligant prostate tissues. It was shown to represent very useful diagnostic marker and also potential therapeutic target for prostate cancer. Two forms of the enzyme were identified in the prostate: full-length transmembrane form consisting of 750 amino acids and a truncated form (called PSḾ), believed to represent spliced variant of PSMA. The cDNAs of both forms are identical except for 266-nucleotide region near 5́end of PSMA that is absent in PSḾ. This deleted region codes for signal peptide as well as for intracellular and transmembrane domains. We are able to detect two protein forms in prostate cancer model cells (LNCaP cells) and we also show that both forms are glycosylated suggesting that this truncated form might originate from the processing of full length transmembrane PSMA. Number of methods including differential centrifugation, pulse-chase experiments, immunochemistry and GFP-fusion protein analysis were used to analyze the origin, cell localization and trafficking of PSMA and PSḾ in the mammalian cells.

B1-022P
Dengue virus NS3 protease: studies on substrate specificity by using internally quenched synthetic peptides

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The NS3 serine protease of dengue virus represents an attractive target for the development of antiviral inhibitors. In this study,

we have investigated the substrate specificity of the NS2B(H)-NS3pro protease by using internally quenched synthetic peptides representing both natural cleavage sequences and their recombinant chimeras. Synthetic peptides incorporating the o-aminobenzoic acid/3-nitro-L-tyrosine fluorescence donor-quencher pair were used to analyze the minimum substrate length requirement, residue preferences and the contribution of prime side residues for enzymatic cleavage by the NS3 protease. A series of peptides derived from the NS3/NS4A cleavage site was designed for the substrate length mapping study. Amino acid truncations in the non-prime and prime side region differently affected rates of substrate hydrolysis and binding as shown by their *K*m and *k*cat values. The optimal substrate identified was a heptapeptide spanning P4–P3'. Chimeric substrates with all possible combinations of non-prime and prime side sequences derived from 5 polyprotein cleavage sites (C, 2A/2B, 2B/3, 3/4A and 4B/5) were assayed for reactivity with the NS3 protease. Kinetic parameters revealed a strong impact of the non-prime side residues on *K*m, whereas variations in the prime side region had greater effect on *k*cat. The fluorogenic derivative of tetrabasic peptide RRRR/GTGN (C/NS5) demonstrated the highest affinity, whereas the peptide KKQR/SAGM (2B/C) had the highest turnover number. The one with the greatest catalytic efficiency was identified as RRRR/SLTL (C/4A). In addition, we have shown that a Ser at P1' is the most preferred residue. The discovery of NS3 substrates with maximized reactivity will be useful for inhibitor development in sensitive high-throughput assays.

B1-023P
Inhibiting the mTOR pathway with CCI-779 results in decreased production of vascular Endothelial Growth Factor in a Head and Neck Squamous cell cancer Cell line

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Introduction: Overexpression of the proto-oncogene eIF4E in surgical margins of head and neck squamous cell cancer (HNSCC) patients is an independent predictor of recurrence and is associated with increase in vascular endothelial growth factor (VEGF) expression. Activation of eIF4E in margins through the mTOR pathway has led us to determine that CCI-779 an mTOR inhibitor has both *in vitro* and *in vivo* growth inhibitory effects in HNSCC cell lines. We wanted to determine if these effects were associated with decrease in VEGF production.
Material and methods: A HNSCC cell line FaDu was treated with 1 and 10 ng/ml of CCI-779 (previously established IC50 = 1 ng/ml). ELISA was used to determine VEGF protein levels in conditioned medium at 30', 1, 2, 4, 6, 24 and 48 h after treatment with the drug and compared to control cells treated with the diluent for each of the time points.
Results: A significant decrease in VEGF production of 70% was noted at 24 h and maintained at 48 h in treated cells when compared to control cells at the same time points. The decrease in VEGF levels (39–47%) was noted within 6 h of treatment with the drug. The percent decrease in VEGF protein levels was the same for both doses of CCI-779.
Conclusions: Overexpression of eIF4E in HNSCC increases translation of mRNAs with long 5'UTRs, one of which is an important angiogenic factor VEGF. Inhibiting the mTOR pathway with CCI-779 can potentially decrease VEGF production. This has future clinical implications for arresting tumor progression in HNSCC patients with molecular positive margins identified by cells overexpressing eIF4E, also known as minimal residual disease.

B1-024P
Proteases from cell culture of *Jacaratia mexicana*

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Plant proteases are important in food industry and food technology. The latex of *Jacaratia mexicana*, Caricaceae, fruits contains a high level of cysteine proteases. In this work was established a cell suspension culture of *J. mexicana*. Callus culture was initiated from stem explants of *J. mexicana* on medium consisted of ¼-strength and full-strength MS mineral salts (Murashige and Skoog, 1965), full-strength MS organics and 6 g/l Agar supplemented with cytokinins: 6-Benzylaminopurine (BAP) at 0.5 mg/l and 6-Furfurylaminopurine (kinetin) at 0.25 mg/l and various concentrations (0.25, 0.5 and 1.0 mg/L) of auxins: 2,4-Dichlorophenoxyacetic acid (2,4-D) 4-amino-3,5,6-Trichloropiridin-2-carboxilic acid (Picloram) Indoleacetic acid (IAA) α-Naphthaleneacetic acid (NAA). All of the treatments induced callus except for the IAA, ANA and without added phytohormones. The best auxin concentration for callus development was determined to be 0.5 mg/l. And the best condition medium for callus development and proteolytic activity of callus was determined to be 0.5 mg/l 2, 4-D + 0.5 mg/l BAP. Cysteine proteases were produced on callus culture of *J. mexicana* and liberated in the medium. Also in the cell suspension culture these enzymes were secreted. Our results support that is possible the synthesis of proteases *in vitro* culture of *J. mexicana*. Since protease is a primary metabolite, further improvement in enzyme production is possible by increasing the growth rate and yield of cell culture of *J. mexicana*.
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B1-025P
High-level expression of human carboxypeptidase M in *Pichia pastoris*. Purification and partial characterization

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Carboxypeptidase (CP) M is an extracellular glycosylphosphatidyl-inositol (GPI)-anchored membrane glycoprotein. This enzyme specifically removes the C-terminal basic residues, lysine

and arginine, from peptides and proteins at neutral pH. It is known to play an important role in the control of peptide hormones, growth factor activity at the cell surface, and in the membrane-localized degradation of extracellular proteins. Therefore, the present work was carried out to clone and express carboxypeptidase M in *Pichia pastoris*, aiming at developing specific inhibitors and to evaluate the importance of the enzyme in different physiological and pathological processes. For this purpose, the enzyme's cDNA was amplified from total placental RNA by RT-PCR and cloned in the vector pPIC9, which uses the methanol oxidase promoter and drives the expression of high levels of heterologous proteins in *Pichia pastoris*. The results show that the CPM gene, after cloning and transfection, integrated in the yeast genome, which started to produce the active glycosylated protein. The recombinant protein was secreted into the medium and the enzymatic activity was measured with the fluorescent substrate dansyl-Ala-Arg. The enzyme was purified by a two-step protocol including gel filtration and ion-exchange chromatography, resulting in a 1761-fold purified active protein in a concentration of 400 mg/l of fermentation medium. SDS-PAGE showed that recombinant CPM migrated as a single band with molecular weight similar to native placental enzyme (62 kDa). These results demonstrate for the first time the establishment of a method using *Pichia pastoris* to express human carboxypeptidase M.

B1-026P
Mutational analysis of active site of glutamate carboxypeptidase II

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Human glutamate carboxypeptidase II (GCP II) is a membrane metallopeptidase expressed predominantly in the nervous system, prostate and small intestine. In the brain, GCP II catalyzes cleavage of the abundant neuropeptide N-acetyl-L-aspartyl-L-glutamate (NAAG) to N-acetylaspartate and glutamate. GCP II is a type II transmembrane glycoprotein with a short cytoplasmic N-terminal region (amino acids 1–18), a transmembrane domain (amino acids 19–43) and a large extracellular domain (amino acids 44–750) where the active site of the enzyme is situated. GCP II, as a cocatalytic zinc metallopeptidase, has two Zn^{2+} ions in the active site which are necessary for its enzymatic activity. Recently, the crystal structure of GCP II was determined in our laboratory and amino acids Arg210, Asn257, Lys699 and Tyr700 were proposed to bind C-terminal glutamate of NAAG (Mesters *et al.*, manuscript in preparation). In the presented study, we carried out site-directed mutagenesis to assess the influence of these amino acid residues on the activity of GCP II. In addition, glutamic acid in the position 424 which is proposed to be involved in proton shift during the catalytical hydrolysis of peptide bond, was mutated to alanine. All the mutant proteins were expressed in insect cells, purified to near homogeneity and enzymatically characterized. It was shown that a mutation in any of these positions lead to significantly reduced NAAG-hydrolyzing activity. The substitution of Glu424 almost completely abolished the enzymatic activity, thus suggesting Glu424 is crucial for enzymatic activity of GCP II. Kinetic characterizations of mutant proteins and their substrate specificities will be presented in comparison with wild type GCP II.

B1-027P
Comparative study of mammalian homologues of human glutamate carboxypeptidase II

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Glutamate carboxypeptidase II (GCPII) is a membrane-bound metallopeptidase. In *Homo sapiens*, GCPII was shown to be expressed in various tissues, mostly in the central nervous system, small intestine and prostate. In brain it hydrolyses N-acetylaspartylglutamate (NAAG), which is the most prevalent peptide neurotransmitter in the mammalian nervous system, to form glutamate and N-acetylaspartate. In small intestine GCPII plays an important role in folate absorption. In prostate its function is still unknown. It was shown that inhibition of GCPII is neuroprotective in many neurodegenerative states. According to current knowledge of this enzyme, its role may also be important in prostate (and possibly other) cancers, where its expression is dramatically changed in comparison with healthy tissue. GCPII is thus becoming an important therapeutic target and diagnostic molecule. In order to analyze structure-activity relationships in related glutamate carboxypeptidases, we set to study the mammalian homologues of human GCPII: GCPII of *Rattus norvegicus*, *Sus scrofa* and *Mus musculus*, which have approximately 90% DNA sequence similarity to human GCPII. Information on the biochemical properties, expression pattern and structural similarity is crucial e.g. for testing of GCPII inhibitors in animal models. We have cloned and expressed recombinant GCPII of *R. norvegicus* and *S. scrofa* in insect cells with the aim to obtain pure recombinant protein sufficient for structural analysis. Data on biochemical comparison of rat, pig and human GCPII forms will be presented and interpreted in the light of the GCPII structure.

B1-028P
Structural analysis of Pla protein from *Y. pestis*: Docking and molecular dynamics of interactions with mammalian plasminogen systemz

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The plasminogen (Plg) system is an important mechanism for the cell migration through the tissues in the mammalian organisms. Some bacterial agents can activate this system by proteases and lead an uncontrolled degradation of extracellular matrix components (MEC), and make an invasive character of these infections. The *Y. pestis* protein Pla is a plasmid coded outer membrane protein, with aspartic-protease activity and is closely related with the proteolytic activation of Plg in the serine-protease form called plasmin. Exactly how the Pla activate Plg in plasmin remains unclear. We performed in this work the predicted interaction between the Plg and Pla protein by rigid-body docking with HEX and evaluate the complex stability by Molecular Dynamics (MD) using the GROMACS. To evaluate the docking accuracy we use the crystal structure of complex Plg-Streptokinase. The MD results show more stability in the docked Plg-Streptokinase complex than in crystal complex observed by the RMSD and RMSF calculations after 2 ns in simulation box. The Pla model

was constructed with Spdb-Viewer using the PDB structure of OmpT as template and quality of model was evaluated with Prochek. The docked complex of Plg-Pla show same interaction site predicted in mutagenesis studies. After 8 ns MD (72 083 atoms in box), we observed the relax of beta barrel structure of Pla and the progressive approximation and stabilization between the cleavage site of Plg into the extracellular loops of Pla, followed of the increase of hydrogen bonds number. In this study we report the possible aminoacids that can be participant in the active site and the sub sites of interaction. The total understanding of these interactions can be a important tool for drug design against bacterial proteases.

B1-029P
Expression of GCPII in human astrocytoma

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Glutamate carboxypeptidase II (GCPII), also known as NAALADase I, folylpolyglutamate hydrolase (FOLH) or prostate specific membrane antigen (PSMA) is localized in number of tissues. In brain astrocytes, it regulates neurotransmission by cleaving neurotransmitter N-acetylaspatylglutamate (NAAG) into N-acetylaspartate and most common excitatory neurotransmitter glutamate. Inhibition of GCPII activity protects against cell death after brain stroke. In animal models it has been also shown that specific inhibitors of GCPII could be useful for the treatment of chronic neuropathic pain, amyotrophic lateral sclerosis and other pathologic situations when excess glutamate is neurotoxic. GCPII is identical to prostate-specific membrane antigen (PSMA), a tumor marker in prostate cancer. GCPII is also found in the membrane brush border of the small intestine where it acts as a folate hydrolase. This reaction expedites intestinal uptake of folate through hydrolysis of folylpoly-gamma-glutamates to monoglutamyl folates. GCPII inhibitors might thus be useful in the imaging and treatment of tumors where folate is required for their growth. Therefore it was of interest to investigate whether GCPII might be upregulated in brain tumors as well. In order to analyze this possibility, we took 57 samples from 49 patients with brain tumors treated in Faculty Hospital Motol during 1999–2004 and determined expression and activity of GCPII by Western blots and immunohistochemistry using monoclonal and polyclonal antibodies developed against extracellular epitopes of GCPII. Moreover, we characterized the enzymatic activity of the enzyme in human samples and correlated the expression of GCPII with the type and grade of the tumor.

B1-030P
Search for optimal isosteres in beta-secretase peptidic inhibitors

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Alzheimer's disease is a widespread, neurodegenerative, dementia-inducing disorder. It is ascribed to the presence of a lesion in several brain regions, the neuritic plaques, which are extraneuronal accumulations of β-amyloid protein (Aβ), a 42-aa insoluble peptide that mixed with axons and dendrites of neurons, interrupt the synaptic process and cause neuronal death. The peptide Aβ is a product derived by proteolitic cleavage from a larger transmembrane cell protein termed amyloid precursor protein, APP. Two enzymes are involved in this cleavage: β-secretase and α-secretase. The first one cuts APP between Met671 and Asp672 of APP to generate the N-terminus of Aβ in the rate limiting step of the process, while the second one cleaves at various places within a sequence between amino acids 712 and 717 to generate the respective C-terminus. Using a combination of molecular modeling techniques, we have designed a set of novel β-secretase peptidic inhibitors with a variety of isosteres starting from the available crystallographic structure of this enzyme bound to the inhibitor OM99-2. Some of the resulting ligands are predicted to have higher affinity for this enzyme than the starting compound. These inhibitors have been synthesized, their β-secretase affinity tested and cell essays have been performed to determine their ability to preclude the formation of Aβ peptides in cell cultures.

B1-031P
NMR studies of human prolyl oligopeptidase: finding new POP peptide inhibitors

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Schizophrenia and bipolar affective disorder (BD) are two neuropsychiatric diseases with high social and economic costs. In spite of the prevalence of these diseases, no effective long-term treatments are currently available. The enzyme prolyl oligopeptidase (POP) shows increased activity in both illnesses. This serine protease hydrolyzes peptide hormones and neuropeptides at the carboxyl end of proline residues. Because of the relevance of POP as a therapeutic target, many specific inhibitors of this protein have been developed in recent years. The inhibitors Ono-1603, JTP-4819 and S-17092-1 are currently in clinical trial phase. S-17092-1 has been administered safely to humans and has been proposed as a potential treatment for cognitive disorders associated with cerebral aging. Our aim is to develop new peptide human POP inhibitors. To obtain the human brain POP required for our studies, the cDNA corresponding to the enzyme was cloned and subsequently expressed in *E. coli*. POP activity was monitored by 19F-NMR using a new synthesized POP substrate labeled with 19F. This substrate allowed us to perform the inhibition assay avoiding the interference problems of colorimetric and fluorimetric assays and was suitable for high throughput screening of new POP inhibitors. Different strategies were used to find putative human POP inhibitors: *in silico* screening and solid phase synthesis of candidates and screening with Chinese medicinal plants extracts. Furthermore, NMR studies were performed with the purified human enzyme by labeling the protein isotopically with 15N and D2O and by selective labeling of the residues methionine and tryptophan with 13C. NMR spectra of the labeled protein were obtained at 800 MHz by applying TROSY techniques. NMR will provide structural information to perform structure-based drug design of new POP inhibitors in the future as well as to study the interaction of the candidates with the active site of the enzyme.

B1-032P
Purification and detection strategies of MT1-MMP from tumor tissue samples

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The crucial regulatory function of the membrane type 1-matrix metalloproteinase (MT1-MMP or MMP-14) in connective tissue metabolism, pericellular proteolysis of extracellular matrix (ECM) components, zymogen activation and angiogenesis was demonstrated with the severe phenotype of the MT1-MMP-deficient mice. This membrane-anchored enzyme is not only essential for normal development of hard tissues, but highly expressed in different human cancers where its level frequently correlates with malignant parameters. In most cases the high level of mRNA or elevated level of protein can be predictive for disease development but these parameters only partly reflect the expression and forms of MT1-MMP in pathological conditions. Biosynthesis, trafficking, intracellular activation, internalization, protein–protein interactions, and the level of physiological inhibitors (TIMPs) strictly influence the activity of MT1-MMP in cells and tissues. In our experimental system, we followed MT1-MMP processing and shedding and characterized the cell-associated and released forms of the enzyme (*JBC* 2000; **275**: 12080–12089; *JBC* 2002; **277**: 26340–26350 and *Biochem J* 2005; **386**: 1–10). We found active and inactive truncated forms of MT1-MMP as a result of treatments or experimentally generated imbalance with TIMPs. We have also developed approaches to identify MT1-MMP forms in tumor tissues. Here we present and discuss different strategies to identify MMP-14 in diverse biological samples. Because MT1-MMP endows tumor cells with the ability to invade and metastasize, these strategies can provide valuable information on the role and function of this key protease.

B1-033P
Contribution of calpain to cellular damage in human retinal pigment epithelium cultured with zinc chelator

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Purpose: We previously showed involvement of calcium-dependent cysteine proteases (calpains, EC 3.4.22.17) in neural retina degeneration induced by hypoxia and ischemia-reperfusion. Aged macular degeneration (AMD) is one of the leading causes for loss of vision. AMD showed degeneration of neural retina due to dysfunction and degeneration of the retinal pigment epithelium (RPE). RPE performs critical functions in neural retina, such as phagocytosis of shed rod outer segments. The purpose of the present study was to determine the contribution of calpain-induced proteolysis to damage in human RPE. Zinc chelator TPEN was used to induce cellular damage since zinc deficiency is a suspected risk factor for AMD.

Methods: Third- to fifth-passage cells from human RPE were cultured with TPEN. Leakage of LDH into the medium was measured as a marker of RPE cell damage. Activity of calpains was assessed by casein zymography, and proteolysis of calpain substrates was detected by immunoblotting. To confirm calpain-induced proteolysis, calpain in homogenized RPE was also activated by addition of calcium.

Results: TPEN caused LDH to leak into the medium from RPE cells, and calpain inhibitor SJA6017 inhibited the leakage. Casein zymography and immunoblotting for calpain and α-spectrin showed activation of calpain in RPE cultured with TPEN. Proteolysis by activated calpain was confirmed by addition of calcium to homogenized RPE.

Conclusion: These results suggested that activation of calpain contributed to RPE damage induced by TPEN *in vitro*.

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B1-034P
In vivo and molecular risk factors of chloroquine or pyrimethamine-sulfadoxine treatment failure in children with acute uncomplicated falciparum malaria

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The risk factors associated with chloroquine (CQ) or pyrimethamine-sulfadoxine (PS) treatment failure were evaluated in 691 children enrolled prospectively in six antimalarial drug trials between July 1996 and July 2004 in a hyperendemic area of southwestern Nigeria. Following treatment, 149 (39%) of 389 children given CQ and 64 (22%) of 302 children given PS failed treatment by day 7 or 14. In a multiple regression model, four factors were found to be independent risk factors for CQ treatment failure at enrolment: age <7 years [adjusted odds ratio (AOR) = 0.46, 95% confidence interval (CI) 0.26–0.84, $P = 0.01$], asexual parasitaemia $>100\ 000/\mu l$ (AOR = 0.46, 95% CI 0.23–0.93, $P = 0.03$), presence of gametocytaemia (AOR = 0.48, 95% CI 0.26–0.88, $P = 0.02$) and enrolment after 4 years of commencement of the study, that is, after 2000 (AOR = 0.47, 95% CI 0.25–0.77, $P = 0.003$). Following treatment with CQ, two factors were independent risk factors for failure of treatment: delay in parasite clearance >3 days (AOR = 0.26, 95% CI 0.1–0.7, $P = 0.014$) and presence of gametocytaemia on day 7 or 14 (AOR = 2.5, 95% CI 1.1–6.0, $P = 0.03$). In those treated with PS, two factors were found to be independent risk factors for PS treatment failure at enrolment: age <1.5 years (AOR = 2.9, 95% CI 1.3–6.4, $P = 0.009$) and presence of fever (AOR = 0.3, 95% CI 0.14–0.78, $P = 0.01$). Following treatment with PS, delay in parasite clearance >3 days (AOR = 0.39, 95% CI 0.18–0.84, $P = 0.016$) was an independent risk factor for failure of treatment. The quintuple mutants made up of triple DHFR (Asn-108, Arg-59 and Ile-51) mutant alleles and double DHPS (Gly-437 and Glu-540) mutant alleles were found in isolates obtained from 33% of patients, was significantly associated with PS treatment failure ($P = 0.001$), while Pfcrt and Pfmdr-1 mutant genes did not significantly predict CQ treatment failure in these patients. These findings may have

implications for malaria control efforts in sub-Saharan Africa where control of the disease depends almost entirely on antimalarial monotherapy.

B1-035P
Development of high-throughput assay of lethal factor using native substrate

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Designing of inhibitors for anthrax lethal factor (LF) is currently of interest as an approach for the treatment of anthrax because LF plays major roles in cytotoxicity of target cells. LF is a zinc-dependent metalloprotease that specifically cleaves the mitogen-activated protein kinase kinase (MAPKK) family. Current assay system for the screening of LF inhibitor use the optimized synthetic peptide coupled with various kinds of fluorophores, which enables fast, sensitive, and robust assays suited to high-throughput screening. However, lines of evidence suggest that the regions beside the cleavage site are also involved in specificity and proteolytic activity of LF. In the present study, we tried to develop high-throughput assay for LF activity based on native substrate, MEK1. The assay system relies on the ECL signal resulting from a specific antibody against the C-terminal region of native substrate. A Glutathione-coated multiwell plate was used as a solid support to immobilize the native substrate by its N-terminal GST-moiety. Immobilized substrate increases the specificity and sensitivity LF-catalyzed substrate hydrolysis compared to the solution phase assay. This assay system would be expected to discover a wide spectrum of anthrax inhibitor.

B2-Protein Degradation

B2-001
The 20S proteasome: mechanisms of assembly and substrate translocation

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While significant progress has been made over the past decade in elucidating the structure and enzymatic mechanism of the 20S proteasome, our understanding of its assembly pathway and the role of the propeptides in the maturation process is still substantially incomplete. Similarly, the mechanisms involved in the translocation of substrates into the central nanocompartment are only dimly understood at present. We have used the Rhodococcus proteasome to dissect the assembly pathway, combining mutagenesis and crystallographic studies. For the thermoplasma proteasome we have established a ''host-guest'' interaction system which allows us to follow the translocation of specific substrates into the interior of the proteasome by electron microscopy, mass spectroscopy and X-ray crystallography.

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B2-002
Transferring substrates to the 26S proteasome in the fission yeast *Schizosaccharomyces pombe*

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The ubiquitin pathway is found in all eukaryotes. In this pathway, target proteins are covalently modified by the addition of ubiquitin, a 76 amino acid protein, to specific lysine residues. The ability of multi-ubiquitin chains to function as a signal to target proteins for degradation by the 26S proteasome is well documented. A key question is how is the multi-ubiquitin chain is recognized as a signal? Fission yeast Rhp23/Rad23 and Pus1/Rpn10 represent two families of multi-ubiquitin chain binding proteins that can associate with the proteasome as well as some E3 ubiquitin ligases. They seem to provide a link to shuttle ubiquitinated substrates from the E3 ubiquitin ligases to the 26s proteasome. A detailed characterization of their proteasome binding will be presented along with their potential role in ubiquitin conjugate dynamics. Finally data will be presented indicating that an additional substrate presentation pathway exists in fission yeast which is also conserved in higher eukaryotes.

B2-003
Non-proteasomal RPN10 raises the threshold for association of a ubiquitin-binding protein with the proteasome

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The ubiquitin proteasome pathway is responsible for the removal of the vast majority of short-lived proteins in the cell. In order to be degraded, a protein substrate is tagged with polyubiquitin and delivered to the proteasome where it is proteolysed. A slew of shuttle proteins is thought to mediate the delivery of polyubiquitinated substrates, although the mechanism remains elusive. One such family of proteins is comprised of Rad23, Dsk2 and Ddi1, which all bind polyubiquitinated substrates through a ubiquitin-associated domain (UBA) as well as the proteasome through their ubiquitin-like domain (Ubl). Another potential shuttle structurally unrelated to the Ubl-UBA family is Rpn10. Rpn10 is found as an integral subunit of the proteasome as well as an in an unincorporated pool. We characterized the interactions of these proteins with individual proteasomal subunits, as well as between themselves. We find unique relationships between the putative shuttle proteins and the proteasome, pointing to functional dissimilarity among them. Strikingly, unincorporated Rpn10 interferes with binding of Dsk2 to the proteasome. Thus, we propose that Rpn10 might play a negative role in proteolysis through its action on Dsk2.

B2-004
Ubiquitin and SUMO as decision makers

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Proteins modified by multi-ubiquitin chains are usually targeted for degradation by the proteasome. In other cases, ubiquitylation mediates protein sorting or regulates other functions. A striking example for a non-proteolytic role of ubiquitin is the RAD6 DNA damage bypass at stalled replication forks. Key elements of this pathway are two ubiquitin-conjugating enzymes, Rad6 and the Mms2/Ubc13 heterodimer, which are recruited to chromatin by the RING-finger ubiquitin ligases, Rad18 and Rad5, respectively. Moreover, also the SUMO-conjugating enzyme Ubc9 is affiliated with the pathway and we discovered that proliferating cell nuclear antigen (PCNA), a DNA-polymerase sliding clamp involved in DNA synthesis and repair, is a substrate. PCNA is (i) mono- ubiquitylated by Rad6/Rad18, (ii) modified by lysine (K) 63-linked multi- ubiquitylation, which additionally requires Mms2/Ubc13/Rad5, and (iii) SUMOylated by Ubc9. All three modifications affect the same lysine residue of PCNA, indicating that they label PCNA for alternative functions. Indeed, we discovered that mono-ubiquitylation of PCNA promotes an error-prone replication bypass, whereas K63-linked multi ubiquitylation mediates error-free replication across the lesions. In contrast, SUMOylation, which occurs even in the absence of DNA damage, prevents recombination between homologs at the replication fork. These findings indicate that mono-ubiquitin, K63-linked multi- ubiquitin chains, and SUMO are crucial for decision making at the replication fork.

B2-005
Plant anaphase promoting complexes: multiple activators and wide-range of substrates keep APC perpetually busy

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Ubiquitin-mediated proteolysis is the primary mechanism in eukaryotes for degrading unwanted and misfolded proteins. Through the cascade of E1, E2 and E3 enzymes, ubiquitin monomers are attached sequentially to the target proteins, which are then recognized and degraded by the 26S proteasome. The selection and specific timing of polyubiquitination of the target proteins are conferred by different E3 ubiquitin ligases. The anaphase-promoting complex (APC) is one of the most extensively studied E3 ubiquitin ligases that plays essential role in the cell cycle and specific developmental processes. The core APC is composed of 11–13 subunits. Except for APC2 and APC11, relatively little is known about the role of the other APC subunits or the assembly of the complex. Two WD40-repeat activator proteins, Cdc20 and Cdh1 determine stage-specific activation of the core APC as well as selection and binding of the APC substrates. In plants, the APC activators are present in multiple copies. *Arabidopsis* contains 5 cdc20 genes, 3 Cdh1-type activators known as ccs52A1, ccs52A2 and ccs52B. Our work has been focused on the function of APC activators in the cell cycle and plant development, identification of novel APC substrates and on the assembly of the APC complexes. APC activities, based on the expression profiles of the cdc20 and ccs52 genes, will be presented at organism level. By detailed protein interaction studies in yeast two hybrid system and *Arabidopsis* protoplasts or transgenic plants, we shall demonstrate how the core APC interacts with the activators and substrates, and propose a model for APC assembly.

B2-006
Characterization of substrate delivery to the *Saccharomyces cerevisiae* proteasome by quantitative shotgun proteomics

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The proteasome is the central protein degradation machinery in the eucaryotic cell. In conjunction with the ubiquitin system, it is responsible for constitutive bulk protein turnover as well as the controlled degradation of regulatory proteins. The system is very well characterized, but the mechanism by which poly-ubiquitinated substrates are delivered to the proteasome remains unclear. Recently our lab has proposed a number of proteins to be proteasome-based receptors for poly-ubiquitinated substrates in *S. cerevisiae* (Rpn10p, Rad23p, Dsk2p; Verma et al., 2004). Others (e.g. Richly et al. 2005) have put forward a complex model for the delivery of substrates from the ubiquitinating machinery to the proteasome involving the AAA ATPase Cdc48p. By analyzing the composition of affinity purified proteasome complexes from *S. cerevisiae* cells lacking these factors and/or exposed to specific proteasome inhibition, we hope to further elucidate the substrate delivery pathway. Ubiquitinated proteins recruited to the proteasome are identified utilizing capillary chromatography in-line to electrospray ion trap mass spectrometry (MudPIT; Link et al. 1999). Using a reference strain grown in minimal medium solely providing heavy Nitrogen (^{15}N) as an internal standard, we are able to record even gradual fluctuations in sample composition. Differences in the recruitment of substrates to the proteasome in varying mutant backgrounds will shed light on the specificity of proteasome substrate receptors and the topology of the substrate delivery mechanism.

B2-007P
Oxidative fragmentation of proteins by a natural antioxidant

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Oxidative protein damage by reactive oxygen species (ROS) produces cross-linking, fragmentation and biochemical modification of the amino acids resulting in biological dysfunctions. Quercetin, a widely distributed bioactive plant flavonoid, possesses anti-cancer, antioxidants and free radical scavenging activities, as well as it binds with DNA causing DNA fragmentation. A little is known about protein oxidative damage and its modifications by antioxidants. Therefore, the aim of the present work was to investigate the molecular mechanisms of antioxidant and pro-oxidant activities of quercetin toward proteins. The antioxidant activities of quercetin, such as superoxide dismutase (SOD)- and catalase (CAT)-mimetic as well as hydroxyl radical (·OH) scavenging activities were possessed. Bovine serum albumin (BSA) was incubated with different concentrations of quercetin. Quercetin has highly SOD- and CAT-like and hydroxyl radical (·OH) scavenging activities. Its activities are concentration dependent. Quercetin fragmentized BSA into specific fragments which they detected by SDS/polyacrylamide gel electrophoresis. Oxidative protein damage was assessed as tryptophan oxidation, carbonyl, quenone and advanced oxidation protein products (AOPP) generation. The increase of protein oxidation products was in concentration dependent manner. The carbonyl and quenone contents and AOPP were highly significantly elevated in querce-

tin-treated proteins when compared with the control sample. The tryptophan fluorescence was highly decreased in treated protein than in the control sample. The mechanisms of antioxidant and pro-oxidant activities of quercetin have been discussed. These results demonstrate that antioxidant quercetin may potentiate protein damage via oxygen free radical generation, particularly .OH radicals by quercetin.

B2-008P
Protein stability mediated by a hyaluronan-binding deubiquitinating enzyme is involved in cell viability

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Protein degradation by the ubiquitin system plays a crucial role in numerous cellular signaling pathways. Deubiquitination, a reversal of ubiquitination, has been recognized as an important regulatory step in the ubiquitin-dependent degradation pathway. We have identified three novel genes encoding a deubiquitinating enzyme, vDUB1, vDUB2, and vDUB3 (villi deubiquitinating enzyme 1, 2, and 3) from human chorionic villi by RT-PCR. Their cDNAs are 1,593 bp in length and encode an open-reading frame of 530 amino acids with a molecular weight of approximately 58 kDa. Expression analysis showed that vDUB transcripts are highly expressed in the heart, liver, and pancreas. In addition, they are expressed in various human cancerous cell lines. Amino acid sequence analysis revealed that they contain the highly conserved Cys, His, and Asp domains, which are required for the formation of active site for the deubiquitinating enzymes. *In vivo* and *in vitro* deubiquitinating enzyme assays indicated that vDUB1, vDUB2, and vDUB3 have deubiquitinating enzyme activity. Here, we show that the overexpression of vDUB proteins leads to irregular nuclear morphology and apoptosis, suggesting that these vDUBs play an important role in regulating signal transduction involved in cell death. Interestingly, the sequence analysis showed that vDUB proteins contain the putative hyaluronan/mRNA-binding motifs, and cetylpyridinium chloride-precipitation analysis confirmed the association between vDUBs and intracellular hyaluronan and RNA.

B2-009P
Selective and mild chemical protein cleavage

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Chemical cleavage of peptide (amide) bonds usually requires harsh conditions. As a result of side reactions and the lack of specificity, chemical amide bond hydrolysis is not a preferred means of protein digestion. We have discovered selective cleavage of peptide bonds in proteins under milder circumstances than any previously reported chemical method. Hydrolysis takes place in aqueous buffers in a pH range of 410, and occurs C-terminal to the proteogenic non-natural amino acid azido-homoalanine (Azhal), effected by a Staudinger reaction after addition of the mild and biocompatible reagent tris(carboxyethyl)phosphine (TCEP). Key feature in the suggested reaction mechanism is the unprecedented nucleophilic substitution of the resulting gamma-iminophosphorane by the flanking C-terminal backbone amide oxygen atom. After hydrolysis, the new C-terminal peptide is present as a homoserine lactone residue and the N-terminal peptide as its free amine. This new reaction may find application as a very mild and selective bio-orthogonal degradation pathway in biochemistry and biomaterials science.

B2-010P
Overexpression of proteasome β5 subunit increases amount of assembled proteasome and confers ameliorated response to oxidative stress and higher survival rates

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The proteasome is the major cellular proteolytic machinery responsible for the degradation of both normal and damaged proteins. Proteasomes play a fundamental role in retaining cellular homeostasis. Alterations of proteasome function have been recorded in various biological phenomena including aging. We have recently shown that the decrease in proteasome activity in senescent human fibroblasts relates to the down-regulation of β-type subunits. In this study we have followed our preliminary observation by developing and further characterizing a number of different human cell lines overexpressing the β subunit. Stable overexpression of the β5 subunit in WI38/T and HL60 cells resulted in elevated levels of other β-type subunits and increased levels of all three proteasome activities. Immunoprecipitation experiments have shown increased levels of assembled proteasomes in stable clones. Analysis by gel filtration has revealed that the recorded higher level of proteasome assembly is directly linked to the efficient integration of ''free''/not integrated β-type subunits identified to accumulate in vector-transfected cells. In support we have also found low POMP levels in β5 transfectants thus revealing an increased rate/level of proteasome assembly in these cells as opposed to vector-transfected cells. Functional studies have shown that β5 overexpressing cell lines confer enhanced survival following treatment with various oxidants. Moreover we demonstrate that this increased rate of survival is due to higher degradation rates following oxidative stress. Finally, as oxidation is considered to be a major factor that contributes to aging and senescence, we have overexpressed the β5 subunit into primary IMR90 human fibroblasts and we have observed a delay of senescence by 45 population doublings. In summary, these data demonstrate the phenotypic effects following genetic up-regulation of the proteasome and provide insights towards a better understanding of proteasome regulation.

B2-011P
Expression levels of the components of the ubiquitin/proteasome pathway in *Pisum sativum* seedlings under anoxia stress

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Oxygen deprivation drastically alters the pattern of protein synthesis in roots. The early response consists of a programmed

change in gene expression: proteins produced under aerobic conditions are no longer synthesized and are replaced by the so-called anaerobic peptides. Among those proteins synthesized under O_2 deficiency some enzymes of the glycolytic and fermentative pathways were identified in plants. Upon reintroduction of air, the anaerobic mRNAs disappear rapidly and the increased levels of those enzymes must return to the basal levels. The ubiquitin/proteasome system is a major pathway of proteolysis in eukaryotic cells and may contribute to controlling the intracellular levels of a variety of short-lived regulatory proteins. In this proteolytic pathway, proteins are covalently conjugated to ubiquitin, which flags them for rapid hydrolysis by the 26S proteasome. Long polyubiquitin chains must be formed to target a protein for destruction by the proteasome. In plants, the ubiquitin-mediated proteolytic pathway is implicated in a variety of cellular processes, including stress responses. In this study, 3-day-old *Pisum sativum* seedlings were subjected to: (i) 15 h of anoxia stress; (ii) 2 h of aerobic conditions after 15 h of anoxia stress and (iii) 4 h of aerobic conditions after 15 h of anoxia stress. The levels of free and conjugated ubiquitin were detected by immunoblotting using anti-ubiquitin polyclonal antibodies. The changes in the mRNA levels of some components of the ubiquitin/proteasome pathway in the seedlings were determined by relative semi-quantitative RT-PCR. The results suggest an involvement of the ubiquitin-mediated proteolytic pathway in the anoxia stress response.

B2-012P
Involvement of the anaphase promoting complex in plant development

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Controlled degradation of short-live proteins via ubiquitin-dependent proteolysis by the 26S proteasome is a key mechanism in eukaryotes that regulates nearly all fundamental cellular processes including cell cycle. Polyubiquitination of the protein substrate is sufficient to target it for degradation by a large ATP-dependent multicatalytic protease, the 26S proteasome. The selection and specific timing of ubiquitination of the target proteins are conferred by different E3 ubiquitin ligase. The anaphase promoting complex (APC) is one of the E3 ubiquitin ligases, which by ordered destruction of various cell cycle proteins has fundamental roles in the regulation of mitotic and endoreproduplication cycles. The APC functions also outside the cell cycle. In post-mitotic cells, the Cdh1 adaptor protein ensures stage specific activation and substrate selection of the APC. In plants, two classes of the Cdh1-type activators have been identified, CCS52A and CCS52B that display differential regulation during the cell cycle and plant development as well as differences in their substrate-specificities. In *Arabidopsis*, transient and complimentary expression profiles of the Atccs52A1, Atccs52A2 and Atccs52B genes indicate APC functions during flower development. To identify APC targets, yeast two hybrid screens were performed in the laboratory. Out of about 200 interacting proteins, several proteins were transcription factors including a key a regulator of flowers development. Data on the interactions of the CCS52 proteins and transcription factors in *Arabidopsis* protoplasts will be presented as well as a model for the APC regulated pathways.

B2-013P
Novel effects of ubiquitin system and chaperone proteins on the prion ''life cycle'' in yeast

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Yeast prion [PSI^+], the self-propagated aggregated isoform of the translation termination factor Sup35, is used as a model system to study neural inclusion disorders. Prion aggregates and other neural inclusions in mammals were previously reported to sequester ubiquitin (Ub). Proteasome inhibitors affected the turnover of mammalian prion proteins. However, a role of Ub-dependent proteolysis in the prion "life cycle" has not been clearly defined. Chaperone proteins, which are also implicated in Ub-dependent proteolysis, have been shown to influence the formation and propagation of the prion aggregates. Our results uncover the connection between alterations of Ub system and chaperone proteins in their effects on the maintenance of yeast prion. We have demonstrated that deletions of genes encoding deubiquitinating enzymes, that are critical for Ub regeneration at the proteasome (Ubp6) or the vacuole (Doa4), cause pleiotropic phenotypic effects that are primarily due to decreased levels of free Ub in the yeast cells. These alterations, as well as deletion of the gene encoding Ub-conjugating enzyme, Ubc4, decreases [PSI^+] curing by the overproduced disaggregase Hsp104, suggesting that Ub system influences Hsp104-dependent clearance of prion aggregates. Spontaneous [PSI^+] formation was also increased in the Ubc4 depleted cells. We previously demonstrated that excess of cytosolic chaperone Ssa of Hsp70 family increases *de novo* formation of [PSI^+]. Both *in vivo* and *in vitro* experiments uncover direct interactions between Sup35 and Hsp70 proteins. The amount of Sup35-bound to Hsp70-Ssa was increased in Ubc4 deletion strain. We propose a model to explain roles of Hsp104, Hsp70 and Ub system in the prion life cycle.

B2-014P
Effects of Parkinson''s Disease mimetics on proteasome activity and protein turnover in human SH-SY5Y neuroblastoma cells

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It has recently been suggested that impairment of the ubiquitin/proteasomal system contributes to the degeneration of dopaminergic neurons (DN) and Lewy body (LB) formation in Parkinson's disease (PD). Mitochondrial dysfunction is also a key factor in PD and agents such as MPP^+ and dopamine, which inhibit mitochondrial electron transport, produce selective degeneration of DN in animal models. In this study the effects of treating SH-SY5Y cells with MPP^+ or dopamine over 72 h on proteasomal chymotrypsin-like activity (CLA) was monitored. MPP^+ (0.1mM) caused a sustained depletion of glutathione levels followed by a reduction in proteasomal activity. A reduction in ATP levels, caused by higher levels of MPP^+ (2mM), exacerbated this effect. Exposure to low dopamine concentrations (0.1mM) led to large reductions in ATP without affecting CLA or glutathione levels; whilst higher concentrations (2mM) caused marked reductions in CLA, glutathione and ATP levels. These results suggest that, under oxidative

stress, glutathione levels are important regulators of proteasomal activity in this cell line. Our group has shown that MPP^+ can destabilize the neurofilament network in SHSY-5Y cells, partly due to changes in phosphorylation of neurofilament (NF) chains. As NFs are important components of LBs, and their mode of turnover is uncertain, we tested the effects of proteasome inhibitors on NF levels. Treatment with these inhibitors led to NF accumulation, which was enhanced when glutathione levels were artificially depleted, suggesting that NFs can be degraded via the proteasomal pathway. The effects of proteasome impairment on protein accumulation will be discussed.

B2-015P
Mitochondria and the hypoxia-inducible factor 1 (HIF-1): regulation of HIF-1 is independent of a functional mitochondrial respiratory chain

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The hypoxia-inducible factor HIF-1 is the "master-regulator" in adaptation to low oxygen concentration and induces the hypoxic expression of several target genes, e.g. erythropoietin and vascular endothelial growth factor (VEGF). In normoxia HIF-1α is constantly produced but also degraded by oxygen-dependent prolyl-hydroxylation. Mitochondria consume most of the oxygen delivered to cells and have been implicated in oxygen sensing. Firstly, mitochondria have been proposed to stabilize HIF-1α by production of reactive oxygen species (ROS) in hypoxia. Secondly, inhibition of the respiratory chain, e.g. by nitric oxide, has been proposed to cause redistribution of intracellular oxygen followed by reactivation of the prolyl hydroxylases and inhibition of HIF signalling. We have used cells depleted of mitochondrial DNA (ρ0) and gas permeable cell culture dishes to eliminate all oxygen diffusion gradients affecting the cells. We show that these dishes neutralize all effects of mitochondrial inhibition. Additionally, cellular hypoxia as assessed by pimonidazole staining has been evaluated in human osteosarcoma cells treated with inhibitors of the respiratory chain under hypoxia. These results demonstrate an elevated pO_2 under hypoxic conditions after treatment with mitochondrial inhibitors correlating with an intracellular oxygen concentration which reduces HIF-1 activation. Thus, neither the absence of ROS nor the redistribution of intracellular oxygen supply leads to the destabilization of HIF-1α in hypoxia. Our experiments provide evidence that an increased intracellular pO_2 evoked by the absence of mitochondrial oxygen consumption reactivates the prolylhydroxylases and is therefore responsible for the degradation of HIF-1α under hypoxic conditions.

B2-016P
Soil protease activity during decomposition of model root exudates released by a model root surface in Cd-contaminated soils

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Enzyme activity is generally higher in rhizosphere than in bulk soil, as a result of a greater microbial activity sustained by toot exudates or due to the release of enzymes from roots. Negative effects of heavy metals on soil microorganisms and enzyme activities have been long recognized. The aim of this study was to assess the stimulatory effects of different low molecular weight organic compounds commonly present in root exudates (MREs) on microbial activity and protease activities and , and how high Cd concentrations affect such stimulatory effects. Soils (Arenic Udifluvent) were sampled from the AGIR long-term field trials, contaminated with Cd nitrate at rates of 0 (control soil), 20 and 40 mg Cd per kg of soil. The MRE solutions contained glucose, citric acid, oxalic acid, glutamic acid or a mixture of the four compounds, added to give a rate of 300 mg of MRE-C per kg of soil. The effects were measured at 4 mm (bulk soil) distance from the MRS. Protease activity was determined by hydrolysis of *N*-benzoylargininamide (BAA). The results showed that different MREs had different stimulatory effects on microbial growth and on the protease activities, mostly localized in the rhizosphere soil layer. In the control soil, the dsDNA content was significantly increased by the addition of all MRE in both rhizosphere and bulk soil layers. The 20 and 40 mg Cd per kg of soil negatively affected on protease activity. The glucose, citric acid, oxalic acid, glutamic acid, MREs mix in both rhizosphere and bulk soil layers, did not stimulate protease. The, microbial growth and protease activities were drastically reduced by high Cd concentrations.

B2-017P
Participation of different digestive proteinases of the yellow mealworm, Tenebrio molitor, in initial stages of hydrolysis of the main dietary protein

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Insects generally have a wide spectrum of digestive proteinases. The knowledge about the impact of different proteinases to initial stages of hydrolysis of dietary proteins is essential for insect control by means of proteinase inhibitors and *Bacillus thuringiensis* toxins. The larvae of a stored grain pest yellow mealworm, *Tenebrio molitor*, were reared on milled oat flakes. The main dietary protein for these larvae was 12S globulin, the main storage protein of oat seeds. To study the initial stages of 12S globulin hydrolysis *in vitro* the reaction was performed in the physiological conditions of anterior midgut (AM) (pH 5.6) by purified enzyme preparations from AM: two fractions of cysteine proteinases Cys II and Cys III, chymotrypsin- and trypsin-like proteinases. Total hydrolysis of 12S globulin was observed with Cys II. Slightly less effective was hydrolysis by chymotrypsin-like enzyme. Cys III cysteine and trypsin-like proteinases produced only partial hydrolysis of seed globulin. In all cases high molecular mass (Mm) intermediate products were formed testifying that hydrolysis of 12S globulin was sequential. Incubation with both cysteine proteinase fractions led to formation of 31 kDa product, while serine proteinases produced 31 kDa and 40 kDa products. Hydrolysis by insect cysteine proteinases resulted in formation of intermediate product similar to the reported earlier single intermediate product formed by cysteine endoproteinase of germinated oats at the beginning of seed germination (Mikola Jones. *Cereal Chem* 2000; **77**: 572–577). Thus formation of intermediate product appears to be a necessary stage in the total 12S globulin hydrolysis.

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B2-018P
Hemorphin: a novel class of regulatory endogenous bioactive peptides derived from hemoglobin degradation. Description of various physiological potential activities

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In contrast to "classical" bioregulator peptides, peptides could be generated in the course of catabolic degradation of functional proteins. For 15 years, we have been interested in such particular group of peptides derived from blood hemoglobin, hemorphins. Hemorphins consist in a family of opioid receptor-binding peptides from 4 to 10 amino acids that are released by proteolytic processing from the (32–41) segment of human hemoglobin beta-chain. They are prevalent throughout the peripheral and central nervous system and have been isolated *in vivo* from tissues or fluids. Many *in vivo* physiological effects have been related (coronaro-constrictory, anti-tumorous, immunoregulatory activities) and several of the hemorphins interact at various levels of the renin-angiotensin system (RAS) by inhibiting angiotensin-converting-enzyme (ACE), aminopeptidase N (APN) and dipeptidyl peptidase IV (DPPIV) activities. In addition, some hemorphins and in particular LVV-Hemorphin-7 (LVVYPWTQRF), binds with high affinity to the brain (IC_{50} = 4.15nM) and renal AT4 angiotensin receptor subtype and is possible the main endogenous ligand from this receptor. In an attempt to characterize *in vivo* precise mechanisms for their release, our attention is focused towards tumoral and central nervous system environments. The last one is particularly interesting as all cellular components implicated in the release of hemorphins are present simultaneously: the haemoglobin precursor and localized brain proteases which might come in contact with blood haemoglobin. In this purpose, the examination of potentiality for this tissue to generate "neuro"-hemorphins would be of interest since sources of hemorphins in the brain have not yet been definitively established.

B2-019P
Expression and subcellular localization of Sgt1 in mammalian cells

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Sgt1 protein, originally discovered in yeast cells, was shown to regulate the activity of kinetochore binding complex CBF3 and ubiquitin ligase complex SCF (Kitagawa et al. *Mol Cell* 1999). Later, we showed that Sgt1 interacts with calcyclin (S100A6) and other calcium-binding proteins of the S100 family (Nowotny et al. *J Biol Chem* 2003). Moreover, in collaboration with Dr Chazin's group, we found that *in vitro* Sgt1 binds to Hsp90 (Lee Y-T et al. *J Biol Chem* 2004). In this work we studied the expression and subcellular localization of Sgt1 in mammalian cells by means of western and northern blots. Among different cell lines examined human embryonic kidney HEK293 and human glioma T98G cells exhibit highest expression of Sgt1 protein. Moreover, we found that in mouse and rat cells there is one isoform of Sgt1, while in human cells two isoforms of this protein were found. To study the subcellular localization of Sgt1 we chose the cells containing moderate level of Sgt1 such as human epidermal Hep-2 cells. By applying immunocytochemistry we found that this protein is present not only in the cytoplasm but also in the nucleus. At present we check the effect of intracellular Ca^{2+} concentration on subcellular localization of Sgt1 and on its co-localization with target proteins.

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B2-020P
Combining reverse genetics, reverse chemogenomics and proteomics to assess the impact of protein N-terminal methionine excision in the cytosol of higher eukaryotes

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In living organisms whatever the cell compartment, proteins are always synthesized with methionine (Met) as the first residue. However, this first Met is specifically removed from most mature proteins. In the course of protein N terminal Met excision (NME), the free N terminal Met is removed by Met aminopeptidase (MAP) cleavage. Three enzymes (MAP1A, MAP2A and MAP2B) have been identified in the cytoplasm of *Arabidopsis thaliana*. By combining reverse genetics and reverse chemogenomics in transgenic plant lines, we have devised specific and reversible switches for the investigation of the role of cytoplasmic NME in *A. thaliana* and of the respective contributions of the two types of cytoplasmic MAP throughout development. In the MAP1A KO context (map1A-1), modulating MAP2 activity by treatment with various concentrations of the specific drug fumagillin impaired plant development. Hence, (i) cytoplasmic NME is essential in plants, (ii) plant MAP1A and MAP2s are functionally interchangeable as a complete block of either MAP type activity does not cause any visible molecular or phenotypic effect, (iii) a minimal level of cytoplasmic MAP is required for normal development and (iv) the plant *A. thaliana* appears an excellent system to study NME and the associated-role of anti-cancer agents like fumagillin. Proteomics was used to assess the impact of NME blocking induced by fumagillin. We used a wild-type plant and the map1A-1 variant grown in the presence of 100 nM fumagillin. The map1A-1 variant showed a dwarf phenotype. We compared by 2D gel electrophoresis the patterns of each protein extracts. Protein spots were identified by tandem mass spectrometry. The data show that fumagillin induces many dedicated pathways, with a prevalence of those related to oxidative stress.

B2-021P
Prolyl endopeptidases from the midgut of the yellow mealworm *Tenebrio molitor*

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Prolyl endopeptidases or post-proline cleaving enzymes are specific endopeptidases hydrolyzing peptide bond on the carboxyl

side of proline residues. These enzymes were found in mammals, several higher plants, fungi and bacteria. It is suggested that the enzymes participate in the *in vivo* regulation of the action of biologically active peptides. We for the first time report about two prolyl endopeptidases in the larval midgut of a stored product pest yellow mealworm *Tenebrio molitor* where they can participate in the proteolysis of one of the main dietary proteins of *T. molitor* larvae – rich in proline prolamines. Characteristics of two prolyl endopeptidases are significantly different. Optimum for hydrolysis of the substrate Z-Ala-Ala-Pro-pNA (N-Carbobenzoxy-L-alanyl-L-alanyl-L-prolyl-*p*-nitroanilide) by prolyl endopeptidase 1 was at pH 8.5, and prolyl endopeptidase 2 – at pH 5.6. Prolyl endopeptidase 1 displayed high pH-stability in the pH range 7.0–10.0 and the rate of hydrolysis increased in the presence of KCl and $CaCl_2$. Prolyl endopeptidase 2 demonstrated low stability in the whole pH range, the rate of hydrolysis strongly decreased in the presence of above mentioned salts, but increased in the presence of high concentrations of EDTA.

B2-022P
The influence of cell growth media on the stability and antitumour activity of methionine enkephalin

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Studies with cultured tumour cell lines are widely used *in vitro* to evaluate peptide-induced cytotoxicity as well as molecular and biochemical interactions. The objectives of this study were to investigate the influence of the cell culture medium on peptide metabolic stability and *in vitro* antitumour activity. The degradation kinetics of the model peptide methionine enkephalin (Met-E, Tyr-Gly-Gly-Phe-Met), demonstrated recently to play an important role in the rate of proliferation of tumour cells *in vitro* and *in vivo*, were investigated in cell culture systems containing different amounts of foetal bovine serum (FBS). The influence of enzyme inhibitors (bestatin, captopril, thiorphan) on the Met-E degradation was also investigated. The results obtained in the Dulbecco's modified Eagle medium containing 10% FBS indicated a rapid degradation of Met-E ($t_{1/2}$ = 2.8 h). Pre-incubation of the medium with a mixture of peptidase inhibitors reduced the hydrolysis of Met-E, as shown by increased half-life to 10 h. The *in vitro* activity of Met-E against poorly differentiated cells from lymph node metastasis of colon carcinoma (SW620) and human larynx carcinoma (HEp-2) cells was determined. Tumour cells were grown 3 weeks prior to the experiment in a medium supplemented with 10, 5 or 2% FBS. Statistically significant to mild or no suppression of cell proliferation was observed in all cultures. In both cell lines, a significant suppression of cell growth by a combination of peptidase inhibitors and Met-E, compared with cells exposed to the peptide alone and cells grown in the absence of Met-E, was observed. This study indicated that caution must be exercised in interpreting the antiproliferative effects of peptide compounds in conventional drug-response assays.

B2-023P
Protein metabolism in whole body and skeletal muscle of laboratory rats treated by proteasome inhibitors

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Proteasome inhibitors are new agents which may be used in treatment of cancer and other severe disorders. One of the possible side effects of their administration is disturbance in protein metabolism which may affect outcome of the illness. Two separate studies were performed using Wistar rats. In the first study, m. soleus (SOL) or m. extensor digitorum longus (EDL) were incubated in medium containing 30 mmol/l MG 132 or 30 mmol/l AdaAhx3L3VS or without inhibitor (control). Protein synthesis was evaluated using L-[1-14C]leucine. Proteolysis was determined according to the rate of the tyrosine release into the medium during incubation. In the second study, proteasome inhibitor MG 132 diluted in dimethyl sulfoxide (DMS) was administered intraperitoneally in dose 10 mg/kg b.w. Controls consisted of DMS treated animals. Changes in protein and amino acid metabolism were estimated in steady-state conditions using continuous infusion of L-[1-14C]leucine 2 h later. Mann-Whitney (*in vivo* study) and paired *t*-test (*in vitro* study) were used for statistical analysis. In *in vitro* study, both MG 132 and AdaAhx3L3VS significantly decreased protein synthesis and proteolysis. However, in *in vivo* study, a significant increase in whole-body protein synthesis and proteolysis were observed in MG 132 treated animals.
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B2-024P
Bioinformatical evidence for a prokaryotic ubiquitin-like protein modification system

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Until recently, the ubiquitin system has been considered a purely eukaryotic invention. By now, the bacterial MoaD/MoeB and ThiS/ThiF systems are known to be prokaryotic versions of a rudimentary activation system for ubiquitin-like proteins. However, similarities to the ubiquitin system end after the activation step, as MoaD and ThiS are not conjugated onto target proteins but rather have a role in the biosynthesis of molybdopterin and thiamin, respectively. The eukaryotic protein Urm1 is the closest homolog of MoaD and ThiS. Unlike its bacterial cousins, Urm1 is conjugated onto target proteins and thus can be considered the founding member of the diverse eukaryotic ubiquitin family. By using a bioinformatics approach that integrates methods of sequence analysis, phylogenetics, phylogenomics and gene-order analysis, we were able to show that many bacteria possess a third ubiquitin-like activation system that most likely is used for protein modifications. The novel system uses a MoaD/ThiS relative, which is more closely related to Urm1 than the typical MoaD and ThiS proteins. These bacterial Urm1 (bUrm1) proteins typically require the proteolytic removal of a C-terminal extension, which masks the GG motif important for activation. Many bUrm1 operons contain a MPN+/JAMM domain protein (belonging to a bona fide ubiquitin-specific protease family), which is most likely responsible for this cleavage. As a third component, an E1-like enzyme is also part of typical bUrm1 operons.

The bUrm1-associated E1 enzymes look more like Uba4 (the eukaryotic Urm1-E1) than like the bacterial MoeB/ThiF E1 enzymes. Interestingly, the MPN+/JAMM protease is also conserved in those bacteria whose bUrm1 end with GG, suggesting that bUrm1 removal is important not only for the activation step

B2-025P
Non-hypoxic induction of hypoxia-inducible factors by insulin and 2-deoxy-D-glucose

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Hypoxia-inducible factors (HIFs) are key mediators of the cellular adaptation to hypoxia, but also respond to non-hypoxic stimuli like insulin. To clarify involvement of all known HIF subtypes in conditions resembling diabetes, we determined distribution of mRNAs and proteins in rats subjected to *in vivo* hypoglycemia and glucoprivation. Wistar rats were infused with either saline, insulin, or 2-deoxy-D-glucose (2-DG) to provoke hypoglycemia or impaired glucose assimilation. Using real-time qPCR, mRNA levels of HIF subunits 1α, 2α, 3α, 1β, and of the target gene GLUT-1 were determined in various organs. Cellular distributions of HIF-α proteins were examined by immunohistochemistry. Treatments with insulin or 2-DG resulted in a widespread increase in HIF-3α mRNA after 6 h, whereas mRNA expression of other HIF subunits remained unaffected, except for HIF-2α which increased in lung and heart after 2-DG. In cerebral cortex and kidney, enhanced staining of all HIF-α proteins was observed after insulin or 2-DG treatments. Lung, heart and kidney showed enhanced levels of GLUT-1 mRNA. Both hypoglycemia and glucoprivation provoke functional activation of the HIF system, with transcriptional up-regulation of HIF-3α representing a typical response. Our data indicate an involvement of the HIF system, and HIF-3α in particular, in the pathophysiology of diabetes.

B2-026P
Fragments of human salivary statherin and PB peptide underlying a furin-like pro-protein convertase action in the pre-secretory salivary fragmentation pathway

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The recent analysis of some derivatives of human salivary peptides and proteins [13], such as acidic and basic proline-rich proteins (PRP) and histatins, allowed recognizing in the pre-secretory salivary fragmentation pathway the action of a furin-like pro-protein convertase of the kexin-subtilisin family, often followed by a carboxy-peptidase action. On the same line, the present study was carried out to search in human saliva the fragments generated from statherin and PB peptide by the action of furin-like proteinases, utilizing a selected-ion monitoring strategy based on HPLC-IT MS. The fragments and post-translational derivatives detected with high frequency in multiple samples were the following: (i) statherin (5380 amu), des-Phe-43 (5232 amu), des-Thr-42-Phe-43 (5131 amu), des-Asp-1 (5265 amu), monophosphor. (5300 amu), statherin SV2 (missing 6–15 residues; 4149 amu), fragm. 10–43 (4128 amu), fragm. 11–43 (3971 amu), fragm. 14–43 (3645 amu). Moreover, the fragm. 6–57 (5215 amu) of PB peptide (5793 amu) was identified. The quantity of these fragments in salivary samples was usually $<10\%$ of the parent peptide. The identified fragments confirmed the action of a pro-protein convertase on furin-like consensus sequences, being the cleavage at Arg- 9 (EKFLR), Arg-10 (LRR) and Arg-13 (RRIGR) for statherin, and at Arg-5 (RGPR) for PB peptide. Detection of statherin missing N- and C-terminus residues indicated also a pre-secretory exopeptidase action, already observed in other salivary peptides. The function of these statherin and PB derivatives in the oral cavity must be elucidated.

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B2-027P
Cloning and expression of a pepstatin insensitive acid protease from *Thermoplasma volcanium* in *E. coli*

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Acid proteases, commonly known as aspartic proteases, are recognized by their specific inhibition by pepstatin. Acid proteases are found in microorganisms both as intracellular and extracellular enzymes. There is very limited number of thermostable, pepstatin insensitive acid proteases isolated from bacterial sources. The only example of purified and cloned acid protease from archaebacteria is thermopsin, produced by *Sulfolobus acidocaldarius*. This thermophilic enzyme represents a new class of acid proteases. To extend our knowledge on the microbial acid proteases with thermostable properties, in this study we have undertaken the cloning and expression of a thermostable, pepstatin insensitive acid protease from themoacidophilic archaeon *Thermoplasma volcanium*. A primer set was designed based on nucleotid sequence of the predicted thermopsin gene and PCR amplification produced a 3080 bp fragment, which covered complete thermopsin gene with some upstream and downstream sequences. The amplified thermopsin gene was cloned in *E. coli*, using pDrive vector. The alignment of the amino acid sequences of thermopsins from various Archaea revealed the highest homology (44%) between the *Tp. volcanium* thermopsin and putative *Tp. acidophilum* enzyme, thermopsin 1. There was a low degree of similarity (28%) between the *Tp. volcanium* thermopsin and thermopsin from *Sulfolobus acidocaldarius*. Expression of the recombinant thermopsin was attempted using QIA Expression KIT, where the cloned gene was ligated to pQE expression vectors to be expressed under the control of T5 promoter. In this system the protein was tagged with 6xHis residue at N-terminal end so that it could be selectively isolated using Ni-NTA metal-affinity chromatography.

B2-028P
Prediction of caspase cleavage sites

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Caspases play an essential role in the execution of apoptotic cell death. Endoproteolytic cleavage by caspases results in either substrate activation or inactivation. Known caspase substrates

include various vital proteins with discrete functions in the propagation of apoptosis. Our aim is to generate a caspase cleavage site predictor specific for each member of the caspase family in order to make subtype-specific predictions of new caspase substrates. We have used a set of experimentally verified proteins to generate sequence logos and train a neural network in order to predict caspase cleavage sites. Machine learning techniques, such as artificial neural networks, are often well suited to integrate the subtleties of sequence variations. This approach also enables integration of structural information in the pattern recognition procedure which could possibly increase the predictive performance of the neural network. The identification of new caspase substrates can lead to further elucidation of several cellular processes involving caspases, including apoptosis, cell cycle regulation, cellular differentiation, and pro-inflammatory responses. In addition, the generation of caspase inhibitors could be greatly aided by a caspase cleavage site predictor.

B2-029P
Regulation of protein synthesis and autophagic-lyososomal protein degradation in isolated pancreatic acini

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A series of biologically active compounds (wortmannin, LY294002, 3-methyladenine, rapamycin, okadaic acid, theophyllin, insulin, glucagon, cholecystokinin) influencing protein synthesis and autophagic-lysosomal protein degradation by interfering with important signalization pathways were investigated. Our results show that in exocrine pancreas cells phosphatidyl inositol-kinases (PI3K-s) are activators, while the target of rapamycin protein (TOR) is an inhibitor of autophagy. cAMP is an inhibitor of lysosomal protein degradation that acts through members of the PI3K family. Okadaic acid inhibits lyososomal protein degradation without inhibiting the formation of autophagic vacuoles. The inhibition of PI3K-s and TOR diminishes protein synthesis, inhibitors of these kinases reduce the synthesis stimulatory effect of insulin. Cholecystokinin showed a biphasic stimulatory effect while glucagon was ineffective on protein synthesis. On the base of these results a possible signalization pathway is suggested for autophagic segregation and lysosomal protein degradation in pancreatic acinar cells.

B2-030P
Purification and characterization of a bifunctional protease from *Vibrio vulnificus*

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Proteolytic enzymes play important roles and are essential factors for homeostatic regulation in both eukaryotes and prokaryotes. In this study, we purified and characterized an extracellular protease showing dual functions as prothrombin activator and fibrinolytic enzyme from *Vibrio vulnificus* ATCC 29307. The purified enzyme had broad substrate specificity towards various blood-clotting associated proteins such as prothrombin, plasminogen, fibrinogen and factor Xa. The cleavage of these proteins could be stimulated by addition of 1 mM Mn^{2+}. The protease could activate prothrombin to active thrombin. However, the thrombin activity generated from prothrombin activation by the protease seemed to be transient, with further cleavage resulting in a loss of activity. Interestingly, the enzyme could enhance the activity of thrombin during the initial rate of fibrin formation when purified fibrinogen was used as substrate. It could also actively digest fibrin polymer as well as cross-linked fibrin. These results suggest that the secreted protease functions as a prothrombin activator and a fibrinolytic enzyme to interfere with blood clotting as part of the mechanism associated with its pathogenicity in human.

B2-031P
Role of the lysosomal cysteine cathepsins and their endogenous inhibitiors in cancer genesis

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Tumor invasion and metastasis are the major causes of treatment failure and death in cancer patients. One requisite for neoplastic cell invasion during tumorigenic processes is the remodeling events that occur within the stroma or extracellular matrix (ECM). Cysteine cathepsins, most likely along with matrix metalloproteases and serine proteases, degradate the ECM, thereby facilitating growth and invasion into surrounding tissue and vasculature. Clinically, the activity levels and localization of cysteine cathepsins and their endogenous inhibitors have been shown to be of diagnostic and prognostic value. The aim of our study was therefore both the determination of prognostic and diagnostic impact of cathepsins B, L and H from human tissues extracts (normal and tumor tissue) and extracellular fluids (such as plasma and urine) and α1-proteinase inhibitor (PI) in pathogenesis of different types of human brain tumors, and extraction and purification of cysteine cathepsin endogenous inhibitors from normal and tumor brains and studying of their physicochemical properties. It was found that the increasing of cysteine cathepsins B, L, H activity levels in brain tumors tissues depend on histostructure, histogenesis and tumor malignancy grade. Increasing of cathepsins L and H activity levels was found in plasma and urine in depending on histogenesis. At the same time decrease in PI activity level was registered. Besides, kinetic characteristics of extracted normal brain endogenous inhibitors of cysteine cathepsins were determined. In extracted tumor brain endogenous inhibitors, there were differences in physicochemical properties in comparison with normal. The data obtained contribute to understanding the participation of cysteine cathepsins and their inhibitors in mechanisms of cancer genesis and both become useful for solving the problem of improving of tumor therapy and provide the possibility of using their activity as diagnostic and prognostic markers.

B2-032P
Protein hydrolysates of sea origin as components for microbiological culture media

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Dry hydrolysate was prepared from protein-containing waste of Icelandic scallop *Chlamys islandicus* processing (SPW) by means of a proteinase complex from king red crabs hepatopancreas. The enzyme consist of the proteolytic enzyme complex from crab hepatopancreas, in which serine proteases dominate (collagenase,

elastase and trypsin- and chymotripsin-like proteinases). As proteinases from king red crab hepatopancreas have high enzyme-substrate affinity to Icelandic scallop proteins, a high degree of proteolysis can be achieved. The composition and properties of the material were investigated on enzymatic protein hydrolysate from SPW obtained under the most technologically suitable conditions: 50–55°C, pH 7.5, 6 h, the ratio between the protein material and the enzyme preparation being 1000:6. For comparison we examined the composition of commercial pancreatic hydrolysate from poor-quality fish species, mainly *Boreogadus* and *Micromestistus*. It was found that hydrolysate from SPW significantly overpowered the commercial analog in the mass percentage of the target product (free amino acid and oligopeptides). The resulting product contains not $<80\%$ free amino acids and oligopeptides. Predominant are aspartic acid, leucine, isoleucine, arginine and lysine, which account for $>5\%$ of the free amino acids. The potential usage of the protein hydrolysate as a nutrient for microorganism cultivation is estimated. Microbiological studies have demonstrated that the hydrolysate from SPW can be used as a protein component in nutrient media. The tested microbial strains satisfactorily grew on the media.

B2-033P
Two functionally distinct protein quality control pathways for degradation of soluble vs. aggregate forms of the Z variant alpha-1 proteinase inhibitor: implications for liver disease in a subset of patients with anti-trypsin deficiency

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The Z variant alpha-1 proteinase inhibitor (A1PiZ) misfolds in the endoplasmic reticulum (ER) and is a substrate for ER-associated protein degradation (ERAD). We report here that A1PiZ degradation is also dependent on VPS30/ATG6, a gene that encodes a component of two PI3-kinase complexes that regulate membrane traffic; complex I is required for autophagy, complex II is required for the CPY-to-vacuole pathway. To elucidate why Vps30p participates in A1PiZ degradation, we tested the hypothesis that ERAD was saturated at elevated levels of A1PiZ expression and that excess A1PiZ was targeted to one of these alternative quality control pathways. Overexpression of A1PiZ led to vacuole-dependent degradation and both complexes were required for delivery of the excess A1PiZ to the vacuole. When the CPY-to-vacuole pathway was compromised A1PiZ was secreted and the distribution of soluble vs. aggregated forms of A1PiZ was comparable with that of wild type yeast. However, disruption of autophagy led to an increase in levels of aggregated A1PiZ; suggesting that when ERAD is saturated the excess A1PiZ is selectively targeted to the vacuole via the CPY-to-vacuole sorting pathway, while excess A1PiZ that forms aggregates in the ER is targeted to the vacuole via autophagy. Together, these results reveal multiple pathways for recognition and removal of aberrant proteins and provide direct evidence that aggregated A1PiZ is removed by autophagy. Our findings may have application in the understanding of, and treatment for, individuals with liver disease caused by the accumulation of ER aggregates of A1PiZ.

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B2-034P
Yeast and lactobacillus association generates peptides from acid goat whey proteins fermentation

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Our goal was to produce peptides from fermentation of unsupplemented acid goat whey by dairy micro-organisms. We used a lactobacillus, *Lactobacillus paracasei*, and a yeast, *Candida parapsilosis*, both previously isolated from a cheese microflora. When co-cultivated aerobically, both micro-organisms grew on unsupplemented goat whey and led to a medium acidification from 6 to pH 3.5. Reversed phase (RP)-HPLC analysis revealed a total alpha-lactalbumin hydrolysis after 96 h of fermentation, a modification of the beta-lactoglobulin elution peak, and 2.5-fold increase in peptide level compared with the non-fermented whey. In the absence of *C. parapsilosis*, *L. paracasei* grew poorly on whey and only a weak medium acidification from 6 to 4.5 was observed after 192 h of fermentation. RP-HPLC analysis revealed a weak modification of beta-lactoglobulin elution peak, a truncated form of alpha-lactalbumin and no peptide generation. *C. parapsilosis* was able to grow on unsupplemented goat whey without modifying pH of the medium, but only 25% of proteins were hydrolysed (alpha-lactalbumin) or denaturated (beta-lactoglobulin) and, again, no peptides were detected. These results suggest that (i) *C. parapsilosis* is required for *L. paracasei* growth and (ii) the co-culture of both micro-organisms is needed to generate peptides from alpha-lactabumin hydrolysis. During co-culture on whey, the use of penicillin G and cycloheximide as bacterial and yeast growth inhibitors respectively, revealed that *L. paracasei* growth was required for medium acidification to pH 3.5 and alpha-lactalbumin hydrolysis. However, we demonstrated that the protease(s) responsible of alpha-lactalbumin hydrolysis was (were) synthesized by *C. parapsilosis* during the first stage of fermentation and that medium acidification (obtained either by *L. paracasei* growth or chemically) was required for yeast protease(s) activity.

B2-035P
Structural characterization by NMR spectroscopy and limited proteolysis of the active form of the NS3 proteinase domain of dengue virus

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Dengue virus causes widespread human diseases such as dengue fever, dengue hemorrhagic fever and dengue shock syndrome. The viral genome is a positive RNA strand that encodes for a single polypeptide precursor. Processing of the polyprotein precursor into mature proteins is carried out by the host signal peptidase and by NS3 serine protease. The three dimensional structure of NS3 protease domain [1–185] NS3pro has been elucidated [1]. Recently a new construct of the recombinant form of the NS3pro, was engineered [2]. We have expressed in *E. coli* the His-tag-CF40.gly.NS3pro protein a new construct of the recombinant form of the NS3pro linked to a 40 -residue

co-factor, corresponding to a part of NS2B, via a non-cleavable, flexible non-apeptide ($Gly_4SerGly_4$), and have currently optimized the purification procedure. Chemically optimized substrates, peptides and depsipeptides, were designed and tested to afford an efficient *in vitro* activity assay, using HPLC and FRET spectroscopy. The data suggest that the amino-terminal region of the 40-amino acid co-factor domain is involved in additional charged interactions with NS3 that are essential for activity as previously described. This form showed catalytic activity and spectroscopic studies were performed to identify the folding of the protein. Moreover, experiments of limited proteolysis have been performed to identify the essential enzymatic domain of the protein and to stabilize the role of the cofactor in the activity and in folding stabilization of the enzyme. After 2 h of the limited proteolysis with endoproteinase Asp-N the product was analyzed by SDS-PAGE and activity assay, showing a high reduction of the molecular mass and only a loss of the activity of the 20%. CD and ^{15}N-^{1}H-HSQC spectra of this protein fragment were performed and other functional and structural characterizations are in progress in our laboratory. It is intended to obtain the structure in solution of the essential active domain of the uniformly ^{13}C,^{15}N-labeled CF40.gly.NS3pro by high-field 3D NMR spectroscopy. The solution structure of the enzyme will be used to answer yet unresolved questions about the mechanism of action, the role of its cofactor NS2B, and the observed substrate specificity.

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B2-036P
Research of *in vitro* anticancer peptides from fish protein hydrolysates

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Introduction: Fish consumption is associated to nutritional benefits due to the presence of proteins of high biological value, minerals, vitamins and polyunsaturated fatty acids. Most studies concerning the benefits of fish consumption on cancer prevention have focused on fish fatty acids but little is known about the potential bioactivity of fish peptides. The present study was then designed to assess the antiproliferative activity of various fish protein hydrolysates, in order to further purify and characterize anticancer peptides.

Methods: Twenty-one fish hydrolysates (from seven species) produced within the framework of the European Valbiomar Programm. Fish hydrolysates composition (protein, fat and salt content) was determined by standard methods (Kjehldhal, Soxhlet extraction and Volhard respectively). Cytotoxic and antiproliferative activity were assayed *in vitro* on MCF-7/6 and MDA-MB-231 human breast adenocarcinoma cell lines, following a cell viability colorimetric assay (Promega, France). Antiproliferative activity of fish hydrolysates was compared with that of reference anticancer molecules with various cellular targets, namely actinomycine D, cytosine-beta-D-arabinofuranoside, cyclophosphamide, etoposide, kenpaullone and roscovitine.

Results: Composition analysis revealed that most hydrolysates contained more than 70% protein. Three Blue Whiting hydrolysates containing 96% protein, 0.5% lipid and 0.2% salt induced a strong breast cancer cells growth inhibition when tested at 1 g/l for 72 h in cell culture medium. Blue Whiting hydrolysates 3, 4 and 5, respectively, induced a growth inhibition of 24.5, 22.3 and 26.3% on MCF-7/6, and 13.5, 29.8 and 29.2 % on MDA-MB-231. These *in vitro* antiproliferative activities are in the range of that observed when the two breast cancer cell lines are treated for 72h with kenpaullone, roscovitine or cytosine-beta-D-arabinofuranoside 10^{-6}M. Further studies are engaged to fractionate and characterize the antiproliferative peptides contained in Blue whiting hydrolysates.

B2-037P
Induction of phenoloxidase activity in the beta-hemocyanin of the gastropod Helix pomatia by limited proteolysis

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Hemocyanins (Hcs) are high molecular mass multidinuclear copper proteins which serve as dioxygen carriers in the haemolymph of several arthropods and molluscs. In recent years, however, also (latent) phenoloxidase (PO) activity has been observed for Hcs, mostly from arthropods, indicating also a possible role in the defence system. Here we report on the PO properties of beta-Hc of the snail *Helix pomatia* (mollusc). This Hc is constituted of 20 identical approximately 450 kDa subunits, each folded into eight approximately 50 kDa functional units (FUs), called Hp a to Hp h and each comprising a dioxygen-binding copper pair (active site). The FUs, liberated from the subunits by limited proteolysis, did not show monophenoloxidase activity with tyramine as substrate nor *o*-diphenoloxidase activity with L-Dopa. With catechol, however, a small intrinsic activity was observed for Hp c > Hp f > Hp h. On further proteolysis with subtilisin at pH 8.2 (20 °C) at an enzyme to substrate ratio of 1/500 (w/w) and a [FU] of 5 mg/ml a strong induction of both monophenoloxidase activity (shown for the first time in a molluscan Hc) and *o*-diphenoloxidase activity was found for FU Hp f. The highest level of induction was reached after 45 h of proteolysis, with a substrate conversion of approximately 17 and 140 nmol/min/mg for tyramine and L-Dopa respectively. After longer times of subtilisin treatment PO activity again declined, indicating that the conformational change, exposing the active site to the substrate, only occurs on limited proteolysis of the FU. For the other FUs no PO induction or only a very slight one (Hp c > Hp h) was observed. The production of fragments was demonstrated by SDS-PAGE. Surprisingly, it was found that FU Hp f is partially resynthesized from split fragments after 37 h, when induced PO activity is at maximum. Atomic absorption measurements showed that nearly no copper was lost from the protein. The induction of PO activity in FU Hp f (and to a much lesser extent in FUs Hp c and Hp h) is accompanied by the appearance of a brownish colour and a concomitant increase in the absorption spectrum around 310 nm, likely to be ascribed to oxidation of tyrosine residues.

B2-038P
Role of the proteasome-mediated proteolytic pathway in laccase production by the white rot fungus *Trametes versicolor* in response to cadmium exposure

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During recent years, it has been established that intracellular proteolysis in eukaryotic cells is largely accomplished by a highly selective non-lysosomal pathway that requires ATP and a large (2.5 MDa) multisubunit complex known as the 26S proteasome. The proteasome-mediated pathway plays vital regulatory functions. It degrades many important proteins involved in cell cycle control, in signaling pathway, and in general metabolism, including transcription factors and key metabolic enzymes. Another function of the proteasomal system is the removal of abnormal, misfolded and oxidized proteins generated under normal and, in particular, stress conditions. To date, proteasomes from other than animal or plant cells were studied only in yeast. Recently, in our laboratory, the proteasome-mediated pathway was shown to be involved in the regulation of ligninolytic activities in the white rot fungi *Trametes versicolor* and *Phlebia radiata* upon nutrient starvation (Staszczak, *Enzyme Microb Technol* 2002; **30**: 537–540). It was the first report on proteasomes in fungi representing Basidiomycota. White rot fungi are able to degrade lignin by the action of secreted enzymes, the best characterized of which are laccases, lignin peroxidases, and manganese peroxidases. The subject of lignin biodegradation has commanded attention for a considerable period of time mainly because of its ecological significance and wide industrial applications of bioligninolytic systems. Heavy metal ions are important environmental pollutants which affect biodegradation processes performed by white rot fungi. In the present study, we investigated whether the proteasomal degradation pathway might be involved in the regulation of laccase production by *T. versicolor* in response to cadmium exposure.

B2-039P
Studies of CacyBP/SIP function using small interfering RNA

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CacyBP/SIP was discovered as a protein that bound calcyclin (S100A6) in a calcium-dependent manner (Filipek and Wojda 1996; Filipek and Kuznicki, 1998) and its distribution and some biochemical properties have been studied. For instance, it has been shown that CacyBP/SIP binds calcyclin via its C-terminal fragment (Nowotny et al. 2000) and that, beside calcyclin, it interacts with other calcium binding proteins of the S100 family (Filipek et al. 2002). Originally, we identified CacyBP/SIP in Ehrlich ascites tumour (EAT) cells but it is also present in other mammalian tissues and cells. In particular, high expression of CacyBP/SIP was found in neuronal cells of mouse and rat brain (Jastrzebska et al. 2000). At present the distribution and structural properties of CacyBP/SIP are quite well described but its function remains obscure. There is only one paper published concerning the possible involvement of CacyBP/SIP in β-catenin ubiquitination and degradation (Matsuzawa and Reed 2001). To elucidate the biological role of CacyBP/SIP we have designed and synthesized siRNA (small interfering RNA) against this protein. This siRNA was then used to transfect neuroblastoma NB-2a and embryonic kidney HEK293 cells, expressing high and low amount of endogenous CacyBP/SIP respectively. The level of CacyBP/SIP was monitored in cell extracts by Western blot technique. We found that siRNA against CacyBP/SIP, which we designed, inhibited the expression of this protein, as its level in transfected cells was lower in comparison with control cells. At present, we checked the effect of diminished expression of CacyBP/SIP on β-catenin degradation and other cellular processes.

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B2-040P
Exposure of *Lemna minor* to arsenite: expression levels of the components of the ubiquitin/proteasome pathway

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Heavy metals are powerful poisons for living cells. It has been shown that exposure to arsenicals, either *in vitro* or *in vivo*, in a variety of model systems, causes the induction of a number of the major stress protein families, such as the heat shock proteins (hsp) (*Toxicol Appl Pharmacol* 2001; **177**: 132). The reasons for heavy metal toxicity *in vivo* are not fully understood, but they are known to contribute to the accumulation of aberrant proteins (BBA,1995,1268, 59). In animal cells, arsenite has been reported to cause sulfhydryl depletion, to generate reactive oxygen species and increase the level of high molecular mass ubiquitin-protein conjugates (*Toxicol Appl Pharmacol* 2003; **186**: 101). In cells submitted to stress conditions, several components of the ubiquitin/proteasome pathway are activated. In this major, eukaryotic proteolytic pathway, multiple ubiquitin molecules are enzymatically ligated to proteins destined for catabolism by an enzyme system composed of three types of enzymes, commonly referred to as E1, E2, and E3. The large ubiquitin-protein conjugates thus formed are subsequently degraded by a very large protease complex, the 26S proteasome, in an ATP-dependent process. The changes in free ubiquitin (Ub) and ubiquitin-protein conjugates (Ub-P) levels were followed by immunoblotting during the incubation of the higher plant *Lemna minor* L.(duckweed) in the presence of arsenite (As), at concentrations known to confer thermotolerance to the plants. The observed increase in the amount of large molecular mass ubiquitin-protein conjugates is indicative of a role for the ubiquitin/proteasome pathway in the response of *Lemna* to As stress. This outcome is primarily attributed to an increased availability in protein substrates during As treatment for three main reasons: an increase in protein carbonyl (a major marker for protein oxidation) content detected by immunoblotting; moderate increments (as determined by semi-quantitative RT-PCR) in the mRNA levels of the codifying sequences for the ubiquitin pathway components: ubiquitin, E1, E2 and the β subunit and the ATPase subunit of the 26 S proteasome; an identical pattern of variation for the large ubiquitin-protein conjugates is observed in the simultaneous presence of As and cycloheximide, indicating that the observed increase in ubiquitin conjugates does not depend on *de novo* protein synthesis.

B2-041P
Ageing and autophagy

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Life of aerobic cells is associated with continuous oxidative damage resulting in the formation of altered, non-functional macromolecules and organelles. Intracellular accumulation of oxidized proteins defective organelles and lipofuscin inclusions are typical manifestations of ageing that preferentially affects long-lived post-mitotic or growth-arrested cultured cells. Autophagy, an important biological mechanism for renewal of damaged intracellular structures, has been found decreased in ageing. To learn more about the role of autophagy in ageing, we studied the effect of the inhibitor of autophagic sequestration 3-methyladenine (3-ma) on human diploid fibroblasts and astrocytes. Inhibition of autophagy in growth-arrested (confluent) fibroblasts for 2 weeks resulted in the accumulation of altered lysosomes displaying lipofuscin-like autofluorescence, especially when 3-ma exposure was combined with hyperoxia. The findings suggest that autophagy is indispensable for normal turnover of lysosomes, and lysosomal components may be direct sources of lipofuscin. The accumulation of oxidatively damaged intracellular structures (so-called biological ''garbage'') was associated with decreased cell viability. Two-week-inhibition of autophagy with 3-ma resulted in a significantly increased proportion of dying cells when compared with both untreated confluent cultures and dividing (subconfluent) cells exposed to 3-ma. Similar results were obtained when autophagic degradation was suppressed by the protease inhibitor leupeptin. The results support the idea that biological ''garbage'' accumulation is essential for ageing and age-related death of post-mitotic cells, which can be prevented by cell division.

B2-042P
Isoform specific degradation of p63 and p73 is mediated by E3-ubiquitin ligases

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Recently two family members of the tumour suppressor gene p53 have been described, p63 and p73, which seem to be necessary for specific p53-induced stress-response pathways. Furthermore, p63 and p73 appears to be crucial to determine the cellular sensitivity to anticancer drugs, particularly in tumours lacking functional p53. Here, we show that p63 and p73 isoforms are also regulated by proteasomal degradation. We have identified several E3-ubiquitin ligases responsible for the regulation of the stability of p63 and p73. We found that the regulation of p63 and p73 is isoform-specific. Furthermore, we demonstrate that ubiquitination of p73 influences the cellular localization of p73 and of the respective E3-ubiquitin ligases. Finally, we show that the expression of the various E3-ubiquitin ligases can be differentially induced by p73-isoforms. In addition, the E3-ubiquitin ligases can influence the apoptotic function of p73. Our findings demonstrate that p63 and p73 are sent to degradation or stabilized by E3-ubiquitin ligases in an isoform-specific manner and we suggest a negative feedback-loop between p63, p73 and their regulators, as they also influence the function of p63 and p73.

B2-043P
Protease activity in blood of healthy donors and breast cancer patients

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Increased level of metalloproteases was shown to accompany tumor angiogenesis and active invasion in adjacent tissue [1]. Development of different types of tumors is often accompanied by increased protease activity in blood [2,3]. In the present study we compared protease activity of plasma and eluate from surface of blood cells in healthy donors and patients with breast tumor. We have demonstrated recently that in blood of healthy donors almost all circulating nucleic acids (cirNA) are bound at the surface of blood cells. In patients with fibroadenoma cirNA were found at cell surface whereas in breast cancer, no cell-surface-bound cirNA were detected in blood [4]. Conjugates of hydrophobic and hydrophilic peptides of CD34 receptor with biotin were incubated with avidin-coated 96-well EIA microplates. Avidinpeptide complex was incubated with samples under investigation and serial dilutions of proteinase K solution, which was used for calibration of protease activity. Undegraded peptides were visualized by incubation with goat anti-peptide antibodies followed by conjugate of anti-goat immunoglobulins with peroxidase. Blood plasma and eluate from surface of blood cells of cancer patients demonstrated increased level of anti- hydrophilic protease activity compared with healthy donors. Increase of protease activity against hydrophilic peptide in blood correlate with decrease of cell-surface-bound cirNA, indicating that blood proteases can affect concentration and distribution of circulated NA.

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B2-044P
Identification of cleavage site and natural substrate specificity of PrtA, a serralysin-type metalloprotease from the entomopathogenic microorganism *Photorhabdus*

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PrtA, a secreted basic metalloprotease of *Photorhabdus*, belongs to the M12B (serralysin) family of proteases. The biological function of these enzymes is not known, but in some cases they are supposed to have a role in virulence. Serralysins are generally assumed to have broad substrate side-chain specificity. Attempts toward the generation of a sensitive and specific substrate of these enzymes had limited success, and no such substrate is available for Prt-A. Through mass spectrometric analysis of PrtA cleavage products of

oxidized insulin A and B chain, we found that PrtA has a well-defined cleavage site preference. Based on this, we developed a sensitive and highly specific oligopeptide substrate through optimization of the amino acid composition and length. The kinetic parameters of PrtA isolated from *Photorhabdus luminescens* ssp. *laumondii* strain Brecon were measured on the best substrate, Dabcyl-Glu-Val-Tyr-Ala-Val-Glu-Ser-EDANS, giving a KM of 8.8×10^{-5}, a kcat of 2.1×10^{-2}/s and a kcat/KM of 2.4×10^{6}. Its poor hydrolysis by various proteases proved its specificity, while it was very sensitivity in measuring PrtA activity in hemolymph samples from *Photorhabdus* infected *Galleria mellonella* larvae. The substrate preference of Prt-A was determined by *in vivo* digestion of hemolymph proteins from *Manduca sexta*. Six minor protein components were selectively cleaved, which were provisionally distinguished under the names PAS-40, PAS-52, PAS-70, PAS-110, PAS-151 and PAS-170. (PrtA Substrate molar mass). The N-terminal amino acid sequence of two PAS proteins, PAS-52 and PAS-110, identified the former as *Manduca serpin*-1, and showed the latter most similar to Jacalin, a lectin-like protein from barley.

B2-045P
Interactions between the serum- and glucocorticoid regulated kinase (SGK), the epithelial sodium channel (ENaC) and the ubiquitin ligases Nedd4 and Nedd4-2

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The epithelial sodium channel (ENaC) is an integral component of the pathway for Na^+ absorption in epithelial cells. ENaC activity is mainly regulated by mechanisms that control its expression at the cell surface, such as ubiquitination. The ubiquitin ligases Nedd4 and Nedd4-2 have both been shown to bind to ENaC and decrease its activity. Conversely, the serum- and glucocorticoid regulated kinase (SGK), a downstream mediator of aldosterone, is able to increase ENaC activity. This effect is at least partly mediated by direct interaction between SGK and Nedd4-2. SGK binds both Nedd4 and Nedd4-2 but it is only able to phosphorylate Nedd4-2. Phosphorylation of Nedd4-2 reduces its ability to bind to ENaC, and hence increases ENaC activity. The impact of the interaction between Nedd4 and SGK remains unclear. Nedd4-like proteins interact with ENaC via their WW-domains. These domains bind PY-motifs (PPXY) present in ENaC subunits. Nedd4 and Nedd4-2 both have four highly homologous WW-domains. Previous studies have shown that interaction between Nedd4 and ENaC is mainly mediated by WW-domain 3. SGK also has a PY-motif, therefore we tested whether the WW domains of Nedd4 and Nedd4-2 mediate binding to SGK. We show that single or tandem WW domains of Nedd4 and Nedd4-2 mediate binding to SGK and that, despite their high homology, different WW domains of Nedd4 and Nedd4-2 are involved. Our data also suggest that WW domains 2 and 3 of Nedd4-2 mediate the interaction with SGK in a concerted manner, and that *in vitro* the phosphorylation of SGK at serine residue 422 increases its affinity for the WW domains of Nedd4-2. The stimulatory effect of SGK on ENaC activity is partly mediated via Nedd4-2 and will decrease if competition between Nedd4 and Nedd4-2 for binding to SGK occurs. We show that Nedd4 and Nedd4-2 are located in the same subcellular compartment and that they compete for binding to SGK *in vitro*.

B3 – Serine Proteases and their Inhibitors

B3-001
Structural and functional relationships between serine-and metallo.carboxy-peptidases and their protein inhibitors

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The concerted or successive action of proteolytic enzymes has been described in a number of important biological processes in which proteins are degraded or matured, such as digestion, turnover (lysosomal, proteosomal...), blood coagulation, developmental remodeling or apoptosis, among others. The complementary action of proteases belonging to different families to achieve a more efficient o a better modulated hydrolytic mechanism is well documented. Specific molecular associations or shared scaffolds between the involved proteases and/or protein inhibitors and defined three-dimensional structures have also been reported. However, only in a few cases such structures involved metallo.carboxy-peptidases or their inhibitors [1]. We shall review this subject and describe, in such a context, a new model found in a marine invertebrate organism in which such a fact takes place. In particular, the characteristics of a novel bifunctional molecule displaying the functionalities and structures of serine- and metallo.carboxy-peptidases will be presented. Its structure is fully different than the ones previously reported by us and collaborative groups for metallocarboxypeptidase inhibitors [2–4].

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B3-002
Regulating the activity of herpes virus proteases

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Herpesviral proteases exist in a monomer-dimer equilibrium in solution. Dimerization is required for activity and a comformational change communicates the oligomerization state of the enzyme to the active site of each intact monomer. Each monomer has an active site, which is spatially separate from the dimer interface. Kaposi's sarcoma-associated herpesvirus (KSHV),

encodes a protease (KSHV Pr), which is necessary for the viral lytic cycle. Like those of other herpesvirues proteases, the dimer interface of KSHV Pr is composed primarily of a helix near the C terminus, of the protein. The helix of one monomer interacts with residues in the symmetrically related helix of the other monomer across the dimer interface as well as with neighboring helices. Small molecule inhibitors, site directed mutagenesis and 2D NMR spectroscopy were used to compare the monomeric and dimeric forms of KSHV Pr and to investigate the relationship of the active site and the dimer interface of the enzyme. Active site inhibition was shown to strongly regulate the binding affinity of the monomer–dimer equilibrium of the protease, shifting the equilibrium completely to the dimeric form of the enzyme. A previously undetermined conformational change provided insight in to the regulation of protease activity by dimerization as well as an explanation for the weak dimerization of a family of enzymes with a disparately large dimer interface compared to their measured binding affinities. Using this information as a guide, protein grafting of the interfacial helix onto a small stable protein, avian pancreatic polypeptide, generated a small macromolecular inhibitor that successfully disrupted the dimer interface and inhibited enzymatic activity. These results provide direct evidence that peptide bond hydrolysis is integrally linked to the quaternary structure of the enzyme, validate the protease as a therapeutic target and suggest the dimer interface may be an alternative site for antiviral design.

B3-003
Proteases and their regulation – from structures to mechanisms and new concepts for intervention

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Proteolytic enzymes catalyze a very simple chemical reaction, the hydrolytic cleavage of a peptide bond. Nevertheless they constitute a most diverse and numerous lineages of proteins. The reason lies in their role as components of many regulatory physiological cascades in all organisms. To serve this purpose and to avoid unwanted destructive action proteolytic activity must be strictly controlled. Control is based on different mechanisms which I will discuss and illustrate with examples of systems and structures determined in my laboratory.

(a) by specific inhibition with natural and synthetic inhibitors
(b) by enzymatic specificity
(c) by activation from inactive precursors accompanied or not by allosteric changes
(d) by co-localization of enzyme and substrate
(e) by cofactor binding accompanied or not by allosteric changes
(f) by controlled substrate processing and access to the proteolytic site

The regulatory principles offer new opportunities of intervention for therapeutic purposes and in crop science.

B3-004
Shape shifting serpins

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The family of serine protease inhibitors known as the serpins is represented in all branches of life and predominate in the higher organisms, including man. They have evolved an extraordinary mechanism to inhibit proteases which distinguishes them from the 20 other families of serine protease inhibitors, and renders them uniquely qualified to control of the proteolytic pathways essential to life. The mechanism is best described as a spring-loaded mousetrap, where nibbling of the peptide loop bait springs the trap and crushes the unsuspecting protease. As with a mousetrap, the active state of a serpin is metastable, and the energy released upon conversion to its more stable form is used to trap the protease. The complexity of the serpin mechanism provides many advantages over the simpler lock-and-key type mechanism, utilized by all other serine protease families. Serpins provide stoichiometric, irreversible inhibition, and the dependence on serpin and protease conformational change is exploited for signaling and clearance. The potential for regulation is also an inherent part of such a complex mechanism, as illustrated by the heparin activation of serpins antithrombin and heparin cofactor II. However, with complexity of mechanism also comes susceptibility to disease causing mutations: both through loss-of-function, as with thrombosis caused by antithrombin deficiency; and gain-of-function, as with dementia caused by neuroserpin polymerization. Many crystallographic structures of serpins have been solved over the past 20 years, and we now have a frame-by-frame cinematic view of the intricate conformational rearrangements involved in protease inhibition, modulation of specificity, and molecular pathology of the remarkable shape-shifting serpins.

B3-005
Structural lessons of serine proteases: function and mechanism of the serine protease-like HGF as a growth factor in Met signaling

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Hepatocyte growth factor (HGF), a plasminogen-related growth factor, is the ligand for Met, a receptor tyrosine kinase implicated in development, tissue regeneration and invasive tumor growth. HGF acquires signaling activity only upon proteolytic cleavage of single-chain HGF into its α/β-heterodimer, similar to zymogen activation of structurally related serine proteases. Although both chains are required for activation, only the α-chain binds Met with high affinity. Recently, we reported that the protease-like HGF β-chain binds to Met with low affinity (Stamos et al. *EMBO J* 2004; **23**: 2325–2335) and that mutational analysis defined a functional Met binding site on HGF β that bears remarkable resemblance to the active site region of serine proteases (Kirchhofer et al. *J Biol Chem* 2004; **279**: 39915–39924). Additional mutagenesis of the β-chain at residues distinct from those in direct contact with the Met Sema domain shows that purified 2-chain HGF mutants also result in impaired Met phosphorylation in A549 cells, cell migration in MDA-MB-435 cells and cell proliferation in BxPC3 cells. This suggests that additional allosterically linked regions may be involved in the signaling process. Furthermore, antibodies directed toward the β-chain or the HGF α-chain result in inhibition of Met phosphorylation in A549 cells. These antibodies also inhibit proliferation in BxPC3 cells and BaF3 cells. Implications for dimerization mechanisms of HGF-dependent Met receptor activation and signaling are presented. In addition, mutagenesis of the HGF β active site region has been investigated with respect to imparting enzymatic activity. Thus while HGF has the function of a growth factor, the structural and receptor binding aspects of HGF are more akin to those of serine proteases.

B3-006
Trypsinogen 4 with a 28 amino acid leader peptide on its N-terminus is the predominant form of the enzyme in human brain

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Gene PRSS3 on chromosome 9 of the human genome encodes, due to alternative splicing, both mesotrypsinogen and trypsinogen 4. Mesotrypsinogen has long been known as a minor component of trypsinogens expressed in human pancreas, while the mRNA for trypsinogen 4 has recently been identified in brain and other human tissues. Analysis of the gene encoding trypsinogen 4 predicted two isoforms of the zymogen: Isoform A may have a 72 amino acid, while Isoform B a 28 amino acid N-terminal leader sequence. The translation initiation site for Isoform A is an ATG codon, while the initiation site predicted for Isoform B is a CTG codon. We measured the amount of trypsinogen 4 mRNA and the quantity of the protein as well in 17 selected areas of the human brain. Trypsinogen 4 could be localized in glial and neuronal cells using immunohistochemical methods. We purified human trypsinogen 4 by affinity chromatography. Our results show that splice Isoform B is the predominant if not the exclusive form of the zymogen in human brain. The N-terminal residue of the isolated protein was identified by amino acid sequencing as a leucine. At the same time the longest mRNA we were able to isolate was barely longer than the one corresponding to splice Isoform B. Although the most trivial explanation of our results is that Isoform A is proteolytically processed to result in Isoform B, it cannot be excluded that leucine rather then methionine is used as translation initiator amino acid.

B3-007P
Search for endogenous substrates for prolyl oligopeptidase in porcine brain

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Prolyl oligopeptidase (PO) is a serine protease present in most tissues, which preferentially cleaves the peptide bond at the carboxyl site of proline residues. The function of PO is unknown, but it has been associated with several disorders of the central nervous system, such as depression and Alzheimer disease. The purpose was to look for endogenous substrates for the recombinant porcine PO in porcine brain. We adapted a method to extract the proteins from the brain with special attention to the smaller polypeptides since PO is not known to cleave peptides larger than 30 amino acids. Subsequently we looked for a method to separate the protein mixture in less complex fractions. 2D-gelelectrophoresis, commonly used in proteomics, is only suitable for proteins with a molecular weight between 10 and 200 kDA and an isoelectric point between 4 and 10. Two-dimensional chromatography offers a suitable alternative for small peptides. We chose ion exchange chromatography as a first and reversed phase high pressure liquid chromatography as a second step. The resulting fractions were divided into two parts. One part was incubated with the purified PO, the other served as a control. By looking for shifts in the mass spectrum between the control sample and the incubated sample, we identified peptides cleaved by PO. Different methods, such as ESI-QTOF-MS and MALDI-TOFTOF-MS, were used to sequence cleaved peptides by MSMS. These experiments allowed us to deduce the sequence requirements for PO cleavage.

B3-008P
Serine protease subtilisin immobilized on novel mesoporous materials

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Serine proteinases are widely used in protein mapping and peptide or ester bond formation. Fixation of enzyme on solid support has many advantages, such as high stability, possibility of recovering and low product contamination by enzyme. Subtilisin Carlsberg, a protease from *Bacillus licheniformis*, was immobilized on mesoporous silica (SBA-15) and several organosilica supports via physical adsorption. The bifunctional mesoporous organosilicas containing CH2–CH2 or CH = CH bridges in combination with organic tethers bearing amino or hydroxyl functionalities were synthesized using supramolecular templating in the presence of non-ionic triblock copolymers and exhibited high surface area and large pore diameters in the range of 50–70 Å suitable for the incorporation of subtilisin. The kinetics of immobilization was examined for six different carriers. It was shown that enzyme retained hydrolytic activity after the immobilization. The dependence of subtilisin loading on the starting concentration of the enzyme during adsorption shows the maximum loading (455 mg protein/g support) at [E] = 20 mg/ml. The pH dependences of loading and activity of immobilized biocatalysts were bell-shaped. For the organosilica support containing amino and hydroxyl groups the pH-dependence was shifted to the alkaline pH by 2 in comparison with the support containing CH2–CH2 bridges. The adsorbed subtilisin desorbs easily in aqueous media, while no leaching of the enzyme was observed in acetonitrile and DMF/acetonitrile mixture (6/4). The immobilized biocatalyst shows high hydrolytic activity after incubation in non-aqueous acetonitrile for 1 week and after 48 h incubation in 60% DMF/acetonitrile mixture. These data indicate a possible application of the obtained biocatalysts in low water media.

B3-009P
Purification, structural and biological characterization of protease inhibitors from *Acacia plumose* seeds

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Protease inhibitors have been used in many current medicines. Therefore, there is a considerable interest inside the pharmaceutical industry in discovering new composites and mechanisms of

protease inhibition, since these investments have led, for example, to new anti-HIV therapeutical tests, coagulation diseases treatment and tests with anti-carcinogenic drugs. Serine protease inhibitors are found in all plant tissues, mostly in the seeds of the Leguminosae subfamilies: Mimosoideae, Caesalpinoideae and Papilionoideae. *Acacia* genus is one of most important member of Mimosoideae, and the presence of protease inhibitors in this genus was described in only three species and none of them were structurally characterized. In this sense, we are studying three new protease inhibitors from *A. plumose* seeds. From saline extract of triturated mature seeds the inhibitors were purified and presented anti-coagulant activity, serine protease inhibitory activity and action on growth of fitopathogenic microorganisms, *in vitro*. The purification steps included size exclusion chromatography on the Superdex-75 column, equilibrated and eluted with PBS, a ionic exchange chromatography on Mono-S (HR 5/5) column, equilibrated with the buffer Sodium Acetate 50 mM (pH 5.0), and eluted with the same buffer in a gradient of 0–0.5 M of NaCl. Three fractions (eluted around 0.18, 0.22 and 0.33 M of NaCl) that presented anticoagulant activity and serine protease inhibition were separated and denoted ApIA, ApIB and ApIC. Their apparent MWs were around 20 kDa, by SDS-PAGE in the absence of reducing agents. In the presence of reducing agents they shown two bands: between 14–22, and 8–6 kDa. The N-terminal analyze of higher MW chains were TYAFL (ApIA); KELLVDNE (ApIB) and TELHDD (ApIC). The circular dichroism spectra of these inhibitors were very similar, presenting a maximum around 230 nm and a minimum in 202 nm, compatible with presence of unordered and beta elements of secondary structure. Their N-terminal, CD spectra and two-polypeptide chains linked by covalent bound, are compatible with Kunitz type inhibitors. Probably these inhibitors are three different isoforms that present different inhibition specificity degree on the serine proteases family. The KI to different Serinoproteases (Trypsin, plasmatic Kalikrein, Elastase, Quimotrypsin) and specificity to the phytopatogenic fungus are being investigated.

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B3-010P
Protein S potentiates the anticoagulant action of recombinant human activated protein C: a comparison between neonates and adults

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Background: Recombinant human activated protein C [rhAPC, Drotrecogin alpha (activated), Xigris™] is a good candidate for the treatment of microvascular thrombosis, disseminated intravascular coagulation, and sepsis in both adults and neonates. Protein S (PS) serves as a non-enzymatic cofactor for the anticoagulant activities of APC. Since levels of PS are physiologically low in neonates (approximately 35% of adult value), a different anticoagulant effect of rhAPC would be expected in neonates compared to adults.

Methods: In the present study we evaluated the anticoagulant action of rhAPC in the presence of various amounts of PS with respect to prolongation of clotting time, suppression of thrombin potential (TP), and suppression of prothrombin fragment 1 + 2 (F1 + 2) generation in (a) PS-deficient plasma spiked to contain increasing amounts of PS (0–140%), (b) in cord plasma containing PS at neonatal level, (c) in cord plasma adjusted to contain PS at adult level, and (d) in adult plasma. Plasma samples were activated by addition of low amounts of lipidated tissue factor (TF).

Results: The anticoagulant efficacy of rhAPC dose-dependently increased in PS-deficient plasma when the PS level was successively raised by addition of purified PS concentrate. Correspondingly, prolongation of clotting time and suppression of both TP and F1 + 2 generation due to addition of rhAPC was significantly more pronounced in cord plasma spiked to contain PS at adult level than in native cord plasma.

Conclusion: PS modulates the anticoagulant action of rhAPC in both cord and adult plasma. The anticoagulant action of rhAPC is significantly more pronounced in adult than in cord plasma due to physiologically higher levels of PS in adults. It can be speculated that in all clinical situations associated with a drop in PS level the anticoagulant efficacy of rhAPC is impaired.

B3-011P
Polyserases, polyproteases with tandem serine protease domains in a single polypeptide chain

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Although the proteases were initially described as enzymes involved in the non-specific degradation of dietary proteins, today it is known that they can also act as highly specific enzymes that perform selective cleavage of specific substrates. Thus, alterations in the structure, regulation or function of this type of enzymes underlie serious human disorders including cancer. To date, more than 550 protease and protease homologs are annotated in man, mouse, and rat genomes (www.uniovi.es/degradome). The increasing complexity of the proteolytic systems has led to the introduction of global concepts as the term degradome to define the complete set of proteases that are produced in a specific moment by a cell, tissue or organism. As part of our studies focused on the characterization of the mammalian degradomes, we have identified and cloned unusual mosaic proteases containing in tandem serine protease domains. The first, called polyserase-1 is synthesized as a transmembrane protein that undergoes post-translational events to generate three independent serine protease domains. The second polyprotease is the polyserase-2, a secreted protein that remains as integral part of the initial protein product. To date, it is difficult to understand the putative functional advantages derived from the complex polyproteases and, albeit extremely unusual, it is not an unprecedented situation. Thus, the amphibians ovochymase and oviductin are polyserine proteases that contain three in tandem serine proteases. In humans, angiotensin-coverting enzyme and carboxypeptidase D are polymetalloproteases that exhibit some similarities to the polyserases. All these polyproteases constitute examples that illustrate an additional strategy for increasing the complexity of the degradomes.

B3-012P
Evolution of a genetic locus, expressing several protease inhibitors with homology to Whey Acidic Protein (WAP)

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We have previously described a locus on human chromosome 20 that gives rise to 14 proteins containing WAP four disulphide core (WFDC) domains. Among them are the elastase inhibitors elafin and secretory leukocyte proteinase inhibitor (SLPI). Both SLPI and elafin are also known to be important components of the innate immune defence by displaying anti-microbial properties. In order to gain a deeper understanding of the biological role of the locus, we have now extended our investigations of its organization and evolution into non-human mammals. Homologous loci were identified on mouse chromosome 2, rat chromosome 3 and dog chromosome 24. Transcript sequences were generated by RACE technology or retrieved from the EST databases. As in humans, the murine and canine loci are divided into two sub-loci separated by approximately 200 kb. The majority of genes are conserved in all species, but the comparison also showed gain and loss of genes, e.g. two human pseudogenes were identified due to the discovery of functional rodent genes, and in the rat several duplications has yielded four SLPI genes. A most interesting finding was that there is no murine elafin gene. The different WFDC domains showed a highly variable species conservation. This was particularly striking in proteins containing multiple domains, where the aminoterminal WFDC generally displayed low conservation, whereas the opposite was true for the carboxyterminal WFDC. The difference could be due to the potential targets of the inhibitors, which might be either highly variable exogenous microbial proteases or conserved endogenous proteases.

B3-013P
Signaling mechanism of thrombin-induced human gingival fibroblast contraction

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Thrombin is activated during gingival tissue injury and inflammation. Thrombin and other bacterial proteases also affect the functions of adjacent periodontal cells via stimulation of protease-activated receptors (PARs). We noted that thrombin and PAR-1 agonist peptide (20 μM) induced the gingival fibroblasts (GF)-populated collagen gel contraction within 2-h of exposure. However, PAR-3 and PAR-4 agonist peptide (< 20 μM) show little effect on collagen gel contraction. U73122 (phospholipase C inhibitor) and 2-APB (IP3 antagonist) were effective in inhibition of GF contraction. Thrombin-induced GF contraction was inhibited by 5 mM EGTA (an extracellular calcium chelator) and verapamil (a L-type calcium channel blocker). In addition, W7 (10 and 25 μM, a calcium/calmodulin inhibitor), ML-7 (50 μM, myosin light chain kinase, MLCK inhibitor), and HA1077 (100 μM, Rho kinase inhibitor) completely inhibited the thrombin-induced collagen gel contraction. Thrombin also induced the phosphorylation of ERK1/ERK2 in GF. However, U0126 only partially inhibited the thrombin-induced GF contraction. Similarly, wortmannin (100 μM), LY294002 (20 μM) (two PI3K inhibitors) and genistein, also showed partial inhibition. Moreover, NAC was not able to suppress the GF-contraction, as supported by slightly decrease in reactive oxygen species production in GF by thrombin. These results indicate that thrombin is crucial in the periodontal inflammation and wound healing by promoting GF contraction. This event is mainly mediated via PAR-1 activation, PLC activation, extracellular calcium influx via L-type calcium channel, and the calcium/calmodulin-MLCK and Rho kinase activation pathway.

B3-014P
Survival of the anticarcinogenic Bowman–Birk inhibitor from soybean at the terminal ileum of cannulated pigs

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Plant protease inhibitors (PI) of the Bowman–Birk class, a major PI class in legume seeds, have emerged as highly promising cancer chemopreventive agents, being capable of preventing or suppressing carcinogenic processes in a wide variety of *in vitro* and *in vivo* animal model systems. In order to exert their chemopreventive properties *in vivo*, plant PI have to resist and survive, at least to some extent, degradation by acidic conditions and digestive enzymes during gut passage. In this study, we have evaluated the survival rate of the Bowman–Birk inhibitor (BBI) in the terminal ileum of cannulated pigs fed defatted soybean. Two different quantitative approaches have been carried out. Firstly, a competitive indirect ELISA assay using an antisera capable to detect BBI free and/or in complex with digestive proteases; secondly, we have carried out spectrophotometric measurements of trypsin and chymotrypsin inhibitory activities in ileal samples, where the presence of BBI metabolites and/or single active loops can be detected. According to the ELISA method, ileal apparent digestibility of BBI was 58 %, which resulted in a recovery of 0.61 mg out of 1.5 mg/kg feed ingested. Significantly higher ileal digestibility values (95 %) were found when trypsin and chymotrypsin inhibitor activities were evaluated. The results suggest that the immunoassay may be overestimating the presence of functional PI by detection of inactive BBI, but also that the presence of complexed BBI with digestive proteases, even if protein extraction was carried out under acidic conditions, could make BBI undetectable in activity assays. Studies are in progress to overcome these drawbacks. The resistance of BBI to the acidic conditions and digestive enzymes of the upper gastrointestinal tract make these proteins very interesting candidates for evaluation as chemopreventive agents, in modulating cell viability and tumor progression within the gastrointestinal tract.

B3-015P
A single amino acid change in a chymotrypsin prevents plant proteinase inhibitor binding

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Plants have evolved economical strategies to combat insects, which on one hand involves the production of multi-domain PIs that can target multiple enzymes with different specificities and on the other, PIs that belong to structurally distinct families. Solanaceous plants, produce both type I and type II families of PIs, which specifically target serine peptidases. This study showed that type I PIs are better inhibitors of a particular class of chymotrypsins within the gut of

Helicoverpa species that is otherwise unaffected by the type II class of inhibitors. Homology models were used to identify a single amino acid substitution in the *Helicoverpa chymotrypsin* that was likely to confer resistance to the type II inhibitor. Our hypothesis was further supported by recombinant expression and mutagenesis of this single amino acid in the type II inhibitor-resistant chymotrypsin. We therefore propose that both type I and II inhibitors are required to protect plants against lepidopteran insects.

B3-016P
Mobility of the sulphate protamin/ low molecular weight heparin complexes in an electrical field

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Glycosaminoglycans low molecular weight heparin (LMWH) activated plasma serine proteases inhibitors. Serine proteases play an important role in thrombogenesis, the process that leads to blood clotting and such as heart attack, stroke and other cardiovascular disorders. LMWH has been used to temporarily render the blood incoagulable during prophylaxis or treatment of thrombosis and sometimes result in serious bleedings and for the heparin anticoagulant activity neutralization used sulphate protamin. It was investigated relationship between new LMWH-SK derivatives (were generated through the controlled cleavage of porcine intestinal mucosa heparin with a mixture of chitinolytic complex from *Streptomyces kurssanovii*) anticoagulant activities and LMWH-SK complexes with sulphate protamin mobility in an electrical field. With this purpose used biospecific electrophoresis in 1% agarose with protamin sulphate. Precipitation zones (zones of the equivalent) in the "rocket" form were generated. Scanning image was saved as jpg format. The "rocket" squares estimated with the help of BandScan program.

Results: LMWH-SK with molecular mass (MM) 14.0; 5.8; 5.4; 4.7; 4.0; 3.4 kD demonstrated antithrombin activities (aIIa) 85–264 IU/mg, activities against factor Xa (aXa) has made 100–278 IU/mg, aXa/aIIa ratio – (0.8–2.2). Correlation coefficients between MM and precipitation zone heights or squares consist 0.56–0.73 ($P < 0.05$), between aXa activities and precipitation zone heights or squares consist 0.37–0.54 ($P < 0.05$).

Conclusion: LMWH-SK was obtained with the chitinolytic complex hydrolisis help has ratio aXa/aIIa-2,2, it is necessary for antithrombotic preparations. With the MM decrease aXa activity increase and precipitation zone heights or squares of the LMWH-SK complexes with sulphate protamin decrease.

B3-017P
On the possible participation of extracellular serine proteases of filamentous fungi in pathogenesis

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The role of extracellular proteases in supplying filamentous fungi with nutrient compounds is well understood and experimentally documented. However there is no definite answer on the question on the need and role of these proteases in pathogenesis. The study of differences in the spectra of extracellular enzymes of saprotrophic and pathogenic fungi performed on *Fusarium* species revealed that activity of secreted serine proteinases of pathogenic *F. culmorum* strain was much higher (up to 20-fold) than that of saprotrophic strain. The use of *F. culmorum* strains differing in pathogenicity (strongly and weakly pathogenic) demonstrated that activity of secreted serine proteases of strongly pathogenic strain was significantly higher (1.5–8-fold) than that of weakly pathogenic strain. This tendency was preserved in calculations of activity towards protein content and dry weight of mycelium indicating on purposeful synthesis and secretion of extracellular proteases by strains with high pathogenicity. At that these differences were much higher when the substrate for trypsin-like proteinases Bz-Arg-pNa was used than in the case of substrate for subtilisin-like proteinases Glp-Ala-Ala-Leu-pNa. According to the data obtained it is proposed that the value activity of trypsin-like proteinases secreted by the fungi correlated with the degree of their pathogenicity and plays, apparently, an important role in pathogenesis.

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B3-018P
Conformational adaptation of a canonical protease inhibitor upon its binding to the target protease increases specificity

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Atomic resolution crystal structure of SGTI in complex with crayfish trypsin provided further data on the molecular basis of the inhibition mechanism of pacifastin type inhibitors. In complex with crayfish trypsin, SGTI exhibits more or less continuous contacts in an extended region (through sites P_{12}–$P_{5'}$) of the molecule. The comparison of this complex with a simulated bovine trypsin-SGTI one shows that more than half of the interaction energy surplus is originated from the extended region of binding. Some of these contacts result from a conformational change of SGTI that was induced by its binding to the enzyme which is strongly supported by the critical comparison of the crystal structure of crayfish trypsin-SGTI complex with the free form of SGTI. Alignment of the NMR structure ensemble with the X-ray structure of complexed SGTI and a careful comparison of the backbone ϑ, ψ angles were carried out. Additionally, NOE-derived restraints and corresponding distances in the complex are also compared. Local conformation of both P_{12}–P_4 and P_4'–P_5' regions of the inhibitor shows significant changes upon binding suggesting that either or both of these regions may act as molecular recognition sites. This comprehensive analysis of the local backbone properties of SGTI in the free and in the complex form made possible to identify conformational similarities and differences responsible for its efficient binding to the enzyme, and provides a good basis for further studying the structural aspects of protease inhibitor specificity.

B3-019P
Kinetic characterization of recombinant human enteropeptidase light chain and its mutant C122S

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As most of serine proteases enteropeptidase light chain contains four disulfide bonds and one nonpaired cysteine at 122 (chymotrypsinogen-derived residue numbering) position which forms disulfide bond linking the pro- and catalytic domains. A mutant of human enteropeptidase light chain Cys122Ser was constructed by site-directed mutagenesis. The recombinant wild type and mutant proteins were produced in *Escherichia coli* BL21(DE3) with expression vector pET-32a. The active proteins were obtained after solubilization and renaturation of the fusion protein thioredoxin/human enteropeptidase light chain from inclusion bodies. After autocatalytic cleavage of thioredoxin the active enzyme was purified on agarose linked soybean trypsin inhibitor. The yield of refolded active enzyme increased from 1.87 to 7.84% in case of Cys122Ser mutant. The wild type and C122S mutant showed similar kinetic parameters for cleavage of small synthetic substrate Gly-Asp-Asp-Asp-Asp-Lys-naphthylamide, small ester thiobenzyl benzyloxi-carbonil-L-lysinate (Z-Lys-SBzl) and fusion protein cleavage. Both enzymes were inhibited by trypsin-like serine proteases inhibitors but not inhibitors of chymotrypsin-like, cysteine- or metallo-proteinases. Recombinant human enteropeptidase light chain and its mutant C122S were active between pH 6 and 9 with a broad optimum at about pH 7.54 and demonstrated quite high stability to different denaturating agents. Both enzymes demonstrated secondary specificity to chromogenic substrate Z-Ala-Phe-Arg-NA with Km = 0.067 mM, $k$cat = 23 s-1.

B3-020P
Proteinaceous low molecular serine protease inhibitors from wood rotting fungi

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Proteolytic enzymes have been firmly established as main regulatory components in a number of cellular and physiological processes. The most important factors influencing the proteolytic enzymes are natural, proteinaceous protease inhibitors, which form complexes with target proteases. They have been extensively investigated from the points of view on physiological functions, as tools for protease enzymology, models for protein–protein interactions and on potential medical applications. There is growing interest in new inhibitors of proteases from various sources. Among known protease inhibitors from fungi are, yeasts inhibitors of proteinases A (asparagine protease) and B (serine protease), and low molecular inhibitors of serine proteinases from fruiting bodies of mushrooms – *Pleurotus ostreatus* and *Lentinus edodes* as well as some undefined proteinase inhibitory activities from water extracts of some species of Basidiomycetes. Searching for new, bioactive metabolites of basidiomycetous fungi we isolated and characterized recently some low molecular, proteinaceous, natural inhibitors of serine proteases, from mycelia of wood rotting fungi – *Trametes versicolor*, *Abortiporus biennis* and *Schizophyllum communae*. Isolation of inhibitors was achieved by ion exchange and size exclusion chromatography. Preliminary characterization of their inhibitory activity (against some serine proteases), pH and temperature optima of action, and molecular mass, were classically analyzed. Analysis of N-terminal amino acid sequences of these inhibitors suggests a new family of serine protease inhibitors from fungi. More detailed characterization of inhibitors (including molecular modeling) and preliminary experiments with laboratory animals and with lines of human cells are in progress.

B3-021P
The role of serine proteases in the lectin pathway of complement activation

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The complement system is a cascade of serine proteases, and mediates essential functions during infection as a part of the innate immunity. Activation of the complement system culminates in the destruction and clearance of invading microorganisms and damaged or altered host cells. Our view about the complement system has changed considerably in the recent years, due to the discovery of a new activation pathway of complement: the lectin pathway. We have recombinantly expressed and characterized the mannose-binding lectin associated serine proteases: MASP-1 and MASP-2. These are related mosaic serine proteases with similar domain organization but with different enzymatic properties. We showed that MASP-2 is capable of autoactivation and it can cleave C2 and C4 complement subcomponents. MASP-2, therefore, can initiate the complement cascade without the contribution of any other protease. We demonstrated that the complement control protein (CCP) modules, which associate directly with the serine protease domain, stabilize the structure of the catalytic region MASP-2 and contain exosites for the large protein substrates. These results are in agreement with the crystal structures of activated and zymogen forms of MASP-2. MASP-1 is the most abundant MBL-associated serine protease but it cannot activate the complement system. We demonstrated that MASP-1 has a more relaxed substrate specificity compared to MASP-2 and the activity of both proteases can be blocked by C1-inhibtor. We concluded that the two MBL-associated serine proteases participate in evolutionary and functionally different pathways.

B3-022P
Comparative kinetic study on S2′ trypsin variants

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By far the most serine proteases have a glycine in position 193, which is part of the S2' subsite (the second subsite on the enzyme surface C-terminal from the scissile bond of the substrate). In contrast, human trypsin 4, the trypsin isoform expressed in human brain, possesses an arginine in that position. The bulky side chain of this amino acid is responsible for the inhibitor resistance, the most striking feature of this isoform, as it interferes with the binding of polypeptide inhibitors to the enzyme surface. A chimpanzee typsin also has an arginine193, while rat trypsin V bears a tyrosine in that position. There is also a snake venom plasminogen activator, a trypsin type serine protease, that contains an S2' phenilalanine. We created glycine, arginine, tyrosine and phenilalanine S2' variants of human and rat trypsins by site directed mutagenesis in order to investigate the effect of these amino acids on the kinetic behaviour. On small chromogenic substrates and synthetic inhibitors, which do not interact with the S2' residue, there is no signifi-

cant difference between the various mutants in catalytic efficiency and inhibitory constants, respectively. However, on oligopeptide substrates the catalytic efficiency decreases 20–50-fold in the non-glycine variants. This effect is even more dramatic with polypeptide partners: the catalytic efficiency drops 200–500 times while inhibitory constants increase by 3–5 orders of magnitude. We conclude that the catalytic mechanism is not fundamentally influenced by the substitution of residue 193, although this amino acid is part of the oxyanion hole. Bulky residues in the S2' subsite hinder mainly the binding to interaction partners.

B3-023P
Structural studies on MASP-2: towards the understanding of the mechanism of autoactivation

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Mannose-binding lectin-associated serine protease 2 (MASP-2), is the key enzyme of the lectin activation pathway of complement, a major element of innate immunity. A dimer of MASP-2 complexed with mannose-binding lectin (MBL) is able to perform its biological functions: upon recognition of the pathogen by MBL MASP-2 undergoes autoactivation, and then initializes the complement cascade by cleaving C2 and C4. MASP-2 is a mosaic protein containing a chymotrypsin like serine protease domain (SP) and further domains with binding sites of MBL or substrates. Our present study focuses on the structural background of the ability of the zymogen form of MASP-2 to undergo autoactivation. We solved the structures of catalytic fragment of MASP-2 both in its zymogen and activated forms. Comparison of the two structures reveals characteristic conformational differences in the classical activation domain and in some other loops lining the substrate binding region. Loop 1 shows a unique conformation with Arg192 blocking the S1 pocket. We docked the activation loop of MASP-2 in the active site of the active enzyme and built a model of the complex of the active and zymogen forms. The model reveals extended regions of molecular recognition. While this model represents the second step of autoactivation (active form cleaves zymogen), the first step (zymogen cleaves zymogen) requires the stabilization of the zymogen enzyme in active-like conformation. We built a model of a zymogen–zymogen complex. Favorable and unfavorable contacts of the two zymogen molecules help us to identify possible molecular switches, as well as contact regions stabilizing an active-like conformation of the zymogen enzyme in the complex.

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B3-024P
Regulation of the Deg2 serine protease in *Arabidopsis thaliana*

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The Deg/HtrA proteases are ATP-independent serine endopeptidases which are present in most organisms, including bacteria, humans and plants. Previous work in our laboratory has shown that the Deg2 protease of the model plant *Arabidopsis thaliana* selectively degrades the photodamaged D1 protein in the reaction center of photosystem II (PSII) *in vitro*. Therefore, Deg2 is thought to catalyze the primary cleavage of photodamaged D1 protein, which is an important step of the repair mechanism that restores functional PSII. Our present studies aim to elucidate the regulation of the Deg2 protease activity, especially with regard to its D1 degrading activity. We found Deg2 associated to the stromal side of the thylakoid membranes and as a soluble protein in the chloroplast stroma. The amount and distribution of Deg2 protein remained unchanged after exposure to different light intensities, which suggest either a substrate regulation or a post-translational regulation of the D1 degrading activity of Deg2. Recent advances on Deg2 regulation and complex formation will be presented.

B3-025P
Novel peptide inhibitors of human kallikrein 2 (hK2)

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Human kallikrein 2 (hK2) is a serine protease produced by the secretory epithelial cells in the prostate. It activates several other proteases that may participate in the proteolytic cascade mediating metastasis of cancer. Thus, modulation of hK2 activity is a potential way of preventing tumor growth and metastasis. Furthermore, specific ligands for hK2 may be potentially useful for targeting and imaging of prostate cancer. We used enzymatically active recombinant hK2 captured by a monoclonal antibody exposing the active site of the enzyme to screen phage display peptide libraries. Six different peptides binding to hK2 were identified using libraries expressing 10 or 11 amino acids long linear peptides. Three of these peptides were specific and efficient inhibitors of the enzymatic activity of hK2. Alanine substitution analysis revealed that motifs of 5–7 amino acid determined the inhibitory activity of the peptides. The peptides are also of potential utility for development of immunopeptidometric assays for hK2, which is promising marker for diagnosis of prostate cancer. Furthermore, these peptides are potentially useful for treatment and targeting of prostate cancer.

B3-026P
The mechanism of autoactivation of the zymogen MASP-2

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The lectin pathway of complement system is an important component of the innate immunity. It provides the first line of defence against infection, since it is activated on the surface of invading pathogens. The activation of the complement system results in the destruction and clearance of foreign microorganisms. Mannose-binding lectin-associated serine protease-2 (MASP-2) is the enzyme which is responsible for the initiation of the lectin pathway of complement activation. MASP-2 is a multidomain serine protease, which is synthesized as an inactive zymogen and become activated upon MBL binds to carbohydrate

residues on the surface of pathogens. We managed to recombinantly express and purify two forms of zymogen MASP-2. One form is the wild type zymogen enzyme, which can be activated, while the other one is a stable zymogen mutant form of MASP-2. We could prepare the zymogen form of wild type MASP-2 under certain conditions which enabled us to examine the kinetics of activation. We demonstrated that activation of MASP-2 is a true autocatalytic activation without the involvement of any other protease. We characterized the enzymatic properties of zymogen MASP-2 using the stable zymogen form. We demonstrated that zymogen MASP-2 cannot cleave small synthetic substrates but it can cleave large protein substrate (C4). A molecular model for the interaction between zymogen and activated MASP-2 during activation has also been built based on the available 3D structures of zymogen and activated MASP-2.

B3-027P
Influence of streptokinase on the fibrinolytic system proteins

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The present study is dedicated to the investigation of the effect of protein by bacterial origin – streptokinase (Sk) on the activity and interaction regulation mechanisms of fibrinolytic system proteins. The study was carried out with use of porcine haemostasis system which plasminogen isn't activated by Sk. Especially we were interested in study of the changing fibrinolytic system parameters such as tissue type plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), plasminogen, α2-antiplasmin activities. Also the main parameters of coagulation system such as fibrinogen, soluble fibrin, fibrin degradation products levels and thrombin activity and quantity were studied. It was used affinity chromatography, electrophoresis, western-blotting, ELISA, determination of proteins activity. It has been determined an increased consumption of plasminogen on 15% in 4 h after streptokinase injection. It was shown that activity and concentration of t-PA were significantly increased in 3.5 times in 1 h. On the next stages of investigation this parameters tend to norm. After Sk injection PAI-1 quantity was increased in two times (16.7 ng/ml compared to normal 8.9 ng/ml). The interesting fact was the activation of prothrombin by Sk without activation of coagulation system *in vivo*. The injection of Sk causes the significant increase of t-PA activity and quantity possibly due to direct or/and indirect effect on endothelial cells. We can conclude that Sk causes PAI-1 secretion due to effect on platelets as 90% of PAI-1 storage is in α-granules of platelets. Thus analysis of the data displayed besides of well-known Sk function the influence of Sk on the changing of fibrinolytic system potential possibly due to its effect on endothelial cells and platelets.

B3-028P
Serine protease LACTB – a component of filament-shaped structures in the mitochondrial intracristal compartments

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Paracrystalline inclusions in the mitochondrial matrix or intermembrane compartment occur in several biochemically unrelated disorders such as myopathies, paragangliomas and steatohepatitis, and in various cell types under normal conditions, as well. However, little is known about the composition of the inclusions, the mechanism of their formation and their relation to disease processes. In this study we have described the helix-shaped structures in the intracristal compartments of rat liver mitochondria that have undergone Ca^{2+}-induced permeability transition. The filaments are anchored in opposing parts of the mitochondrial membranes and appear to support the cristae mechanically. A protein, that apparently is a component of these helical filaments, has been identified as serine protease LACTB. This protein shows close sequence similarity to the class C bacterial beta-lactamases and is the only member of this class in animals. Since LACTB has not been studied previously we cloned its cDNA for expression in *E. coli* as C-terminal His-tagged fusion protein. LACTB underwent proteolytic processing in both *E. coli* and in isolated mitochondria resulting in several protein fragments. This is likely to be due to autocleavage and may be an activation/maturation process. 2D blue native gel electrophoresis indicated that LACTB was part of a >600 kDa protein supercomplex. In summary, the presence of the serine protease motive in LACTB and its supposed ability to form helical filaments suggest that LACTB might function not only as a component of 'mitoskeleton' in maintaining and rearranging the mitochondrial ultrastructure under certain conditions, but also might take part in apoptotic processes.

B3-029P
Novel psychrophylic trypsin-type protease from *Serratia proteomaculans*

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Proteinase with trypsin specificity from psychrophylic microorganism *Serratia proteomaculans* was partly purified. It was shown that the properties of this enzyme (temperature and pH-stability, efficiency of substrate hydrolysis) correspond with the psychrophylic character. Inhibitor analysis and study of substrate specificity indicate that this enzyme is serine trypsin-type protease. At the same time this enzyme is zinc-dependent. Proteases of such type were unknown till now. Secondary specificity of the studied enzyme differs from the bovine trypsin specificity – this protease hydrolyses the short substrates more efficient. Zinc, cadmium (II) and copper (II) ions in mmolar concentrations inhibit the enzyme activity. The unusual character of calcium ions influence on substrate hydrolysis and inhibition by the bovine pancreatic trypsin inhibitor (BPTI) was registered for the studied enzyme.

B3-030P
Activation pathway analysis of Rat-{Delta}{alpha}-chymotrypsin by MD and TMD methods

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The activation of chymotypsinogen into chymotrypsin happens via the proteolytic cleavage of the R15-I16 bond and the subsequent rotation of residue I16 from the solvent into the interior of the protein [1]. As a result, a stabilizing salt bridge between the amino terminus of I16 and the side chain of D194 is formed. The transition from the inactive form with the solvent exposed I16 to the active form with the buried I16 residue can be induced

in vitro by a neutral to basic pH change [2, 3]. The kinetics of the activation process can be followed by Stopped Flow Fluorescence (SFF) experiments while the structural features of the transition can be explored by in silico Molecular Dynamics (MD) and Targeted Molecular Dynamics (TMD) [4] simulations. To challenge the activation process, mutants were constructed and studied by SFF measurements. Subsequently, on these mutants multiple MD/TMD simulations were carried out. Our results indicate the existence of parallel activation pathways. They demonstrate the absolute necessity of multiple simulations and of proper statistics. They reveal the pros and cons of the TMD method.

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B3-031P
Purification and characterization of a serine protease from wheat

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A simple method for the purification of a novel serine endoprotease from wheat *Triticum aestivum* (cv. Giza 164) has been developed. It consists of ion-exchange and gel filtration. The molecular mass of the enzyme was 58 kDa by SDS/PAGE under reducing conditions and 57 kDa by gel filtration on a Sepharose 6B column. The enzyme had isoelectric point and pH optimum at 4.2 and 4.5, respectively. The substrate specificity of the enzyme was studied by the use of synthesized and natural substrates, azocasein, azoalbumin, hemoglobin, casein, gelatin and egg albumin. The enzyme appears to prefer azocasein with *K*m 2 mg azocasein/ml. The enzyme had a temperature optimum at 50 °C with heat stability up to 40 °C. While Co^{2+} and Mg^{2+} accelerated the enzyme activity by 54 and 56%, respectively, Ca^{2+} and Ni^{2+} had very little effect. The enzyme was strongly inhibited by phenylmethylsulphonyl fluoride (PMSF), but not by the other protease inhibitors, suggesting that the enzyme is a serine protease. From the results it can be concluded from the characterization that the *T. aestivum* serine protease may be suitable for food processing.

B3-032P
In vitro effects of a potent, selective Dipeptidyl Peptidase II (DPPII) inhibitor in leukocytes

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Dipeptidyl Peptidase II (DPPII, E.C. 3.4.14.2) is a vesicular protease that releases N-terminal dipeptides from oligopeptides with Pro or Ala in the penultimate position, preferably at acidic pH. Natural substrates of DPPII still have to be elucidated. Quiescent cell proline dipeptidase (QPP), likely to be identical to DPPII [Maes et al. *Biochem J*, in press], has been suggested to be involved in the process of apoptosis [Chiravuri et al. *J Immunol* 1999; **163**: 3092–3099]. DPPIV, a DPPII/QPP related enzyme, is currently investigated as a therapeutic target. In order to investigate the DPPII function, we developed potent and highly selective DPPII-inhibitors. In a first step, we investigated the *in vitro* applicability of the potent DPPII-inhibitor N-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3(S)-butanediamine dihydrochloride (IC50 DPPII: 0.48 ± 0.04 nM ; IC50 DPPIV : 165 ± 9 μM) [Senten et al. *J Med Chem* 2004; **47**(11): 2906–2916] in human peripheral blood mononuclear cells (PBMC) and U937-cells. The compound was able to penetrate the cell membrane and proved efficacy without evidence for acute cellular toxicity. There was a dose-dependent inhibition of intracellular DPPII activity without affecting the DPPIV activity (maximal efficacy at 100 nM). These properties enable to differentiate between DPPII and DPPIV in biological systems and allow further investigation of the physiological function of DPPII. In a second step, we have been investigating the involvement of DPPII in apoptosis in human leukocytes by using this compound. Preliminar results based on annexin V-/PI-staining using up to 1 μM inhibitor in U937-cells and PBMC did not show signs of apoptosis while DPPII activity was inhibited for ~90%.

B3-033P
Regulation of enteropeptidase activity by calcium ions

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Effect of calcium ions on hydrolysis of peptide substrates of general formula A-$(Asp/Glu)_n$-Lys(Arg)-B, catalyzed by enteropeptidase (EC 3.4.21.9), differs depending on substrate type. For specific enteropeptidase substrates (n = 4) calcium ion exhibits the promotion of hydrolysis by the natural two-chain enteropeptidase. Hydrolysis of atypical enteropeptidase substrates (n = 1–2) is as a rule less efficient; in addition calcium ion shows in this case the inhibition influence. Therefore the regulation of the non-desirable side-hydrolysis during full-length enteropeptidase-catalyzed chimeric proteins processing is possible by means of calcium ions. On the contrary the hydrolysis of substrates of all type (n = 1–4) by enteropeptidase light chain as well as the enzyme containing the truncated heavy chain (466–800 or 784–800 fragments) is inhibited by calcium ions. Hydrolysis of the natural enteropeptidase substrate, trypsinogen, is at least two orders of magnitude more efficient than any artificial substrate hydrolysis. We propose that this effect is caused by participation in trypsinogen coordination with enzyme of the addition secondary substrate binding site and/or calcium-binding site; both sites located on the N-terminal half (118–465) of the enteropeptidase heavy chain. One more mechanism of the regulation of the enteropeptidase activity by calcium ion is the unusual calcium-dependent autolysis of the enteropeptidase heavy chain leading to the drastic loss of its activity towards trypsinogen. Autolysis of enteropeptidase heavy chain and well-known autolysis of trypsin were compared; the second one serves as the natural defense mechanism against the undesirable premature proenzymes activation in pancreas leading to pancreatitis. The corresponding enteropeptidase inactivation in low Ca^{2+} ion environment might be the component of the same protective mechanism.

B3-034P
Human trypsin 4 selectively cleaves myelin basic protein: Is this brain protease involved in the pathomechanism of multiple sclerosis?

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Demyelination, the breakdown of the major membrane protein of the central nervous system, myelin is involved in many neurodegenerative diseases. Proteases participating in this process are potential targets of therapy in neurodegenerative diseases. In the present *in vitro* study the proteolytic actions of calpain, human trypsin 1 and human trypsin 4 (the product of gene PRSS3) were compared on lipid-bound and free human myelin basic protein as substrates. Digestions only with calpain and human trypsin 4 actions may be of some physiological or pathological relevance, since these two are expressed in human brain. The fragments formed were identified by using N-terminal amino acid sequencing and mass spectrometry. The analysis of the degradation products showed that human trypsin 4 of these three proteases cleaved myelin basic protein most specifically. It selectively cleaves the Arg80-Thr81 and Arg98-Thr99 peptide bonds in the lipid bound form of human myelin basic protein. Based on this information we synthesized region 94–104 of myelin basic protein, peptide IVTPRTPPPSQ that contains the specific trypsin 4 cleavage site Arg98-Thr99. *In vitro* studies on the hydrolysis of this synthetic peptide by trypsin 4 confirmed our results with intact myelin basic protein. What lends some biological interest to the above finding is that the major autoantibodies found in patients with multiple sclerosis recognize sequence 80–96 of the protein. Our results suggest that human trypsin 4 may be one of the candidate proteases involved in the pathomechanism of multiple sclerosis.

B3-035P
Further biochemical characterization of recombinant catalytic subunit of human enteropeptidase

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Enteropeptidase is a heterodimeric serine protease of the intestinal brush border that activates trypsinogen by highly specific cleavage of its activation peptide following the sequence Asp-Asp-Asp-Asp-Lys. Its light chain alone is sufficient for an effective cleavage of fusion proteins with trypsinogen activation peptide analog. Human enzyme possesses 10-fold specificity coefficient compare to bovine one, and an explanation of this fact can contribute a lot to the attempts of improving or modulating enzymatic properties. Highly pure and active recombinant human enteropeptidase light chain (L-HEP) was obtained by renaturation from inclusion bodies expressed in *Escherichia coli* cells and the active L-HEP was purified on agarose-linked soybean trypsin inhibitor. Enzymatic activity of purified L-HEP was studied through the cleavage of the synthetic peptide substrates and several fusion proteins. L-HEP associated with soybean trypsin inhibitor slowly and Z-Lys-SBzl cleavage was inhibited with K_i^* = 2.3 nM. Comparison of L-HEP and bovine enteropeptidase inhibition by bovine trypsin inhibitor aprotinin has shown almost an order difference in K_i^*. pH dependence of the enzyme activity was measured and pH optimum point was found to be 7.54. Enteropeptidase light chain amino acid sequence and crystal structure were analyzed for the presence of target regions for mono- and bivalent ions. Unlike trypsin with predicted and experimentally proved calcium-binding sites and sodium-activated thrombin, L-HEP was predicted to be deprived of any of such sites and an influence of these ions on the cleavage of different substrates was found to be confined primarily to a substrate binding.

B3-036P
Study of the proteomics changes induced by the interaction of snake venom proteases and their natural inhibitors from *Mucuna pruriens* in mouse plasma

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As a continuation of our efforts to fully elucidate the anti-snake venom properties of *Mucuna pruriens* and to further understand the molecular changes that occurred in mouse plasma proteome as a result of *in vivo* challenge test with venom and *Mucuna pruriens* proteins (MPE), Two Dimensional Polyacrylamide Gel Electrophoresis was done. Plasma was pooled and gels were run in triplicate to eliminate both biological and experimental variations. Analysis using ImageMaster 2D platinum software and other statistical analysis tools showed significant differences in protein expression between all the treatments and the control group. Some proteins were down regulated, some up-regulated, some completely disappeared while new protein spots were identified. The protein expression of plasma of mouse immunized with MPE for 3 weeks before challenge with lethal dose of venom and that injected with venom alone was more complex. Some venom proteins like ecarin are serine proteases that activate clotting factors like prothrombin, causing haemorrhage and disseminated intravascular coagulation, on the other hand, the protease inhibitors from *Mucuna pruriens* must have acted to antagonize these effects by direct proteolysis (cleavage products/spots appearing in the protein map) or other immunological mechanisms. The results obtained represents the first proteomics approach in studying all the plasma proteins involved in this phenomenon. We have only concentrated on protein spots showing interesting variations with respect to control. It is also an important step in the identification of the affected proteins, the kind of modifications/molecular mechanisms involved which is likely the basis of the *in vivo* protection the plant extract showed against the venom.

B3-037P
Characterization, cloning, recombinant expression and protein engineering of a cryophilic protease of marine origin and its engineered mutants

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The use of enzymes at low temperatures has great potential in terms of lower energy costs, therapeutic applications and to lower microbial contamination in industrial processes. Low temperature proteases (cryophilic – or psycrophilic – proteases) are of particular interest for detergents and as wound debriding agents. At present, we are studying cryophilic proteases from Antarctic Krill (*Euphausia superba*), which normally lives in the sea at temperatures near 0 °C. We have isolated several low temperature proteases by chromatography. Enzyme activities and stability were characterized at low temperatures and as a function of pH to find optimum conditions for different applications. A particular enzyme, named KT1, showed particularly high specific activity at 20 °C, several times that of commercial preparations of proteases such as subtilisins. This protein showed a high degree of similarity with digestive trypsins isolated from various arthropoda species. Using mRNA molecules obtained from abdominal sections of *E. superba* and subsequently subjected to a reverse-transcription reaction, we identified, isolated and sequenced a DNA molecule that codes for an inactive zymogen of the enzyme. Cloning of this DNA sequence in *Escherichia coli* strains allowed the recombinant expression of the zymogen, followed by purification and activation of the zymogen, which lead to an active cryophilic trypsin. We performed a homology modeling procedure that conducted us to obtain a molecular model of the mature enzyme. The 3D model thus obtained was refined using energy minimization, hydrogen network optimization and residue–residue contact optimization techniques, leading to a reliable model of the enzyme. We used this model to identify many interesting and novel features of the enzyme molecule that could be related with its cryophilic character, and to propose site-directed mutagenesis strategies that could be used to improve the enzyme performance at low temperatures, its pH-activity profile, specificity, inactivation resistance and recombinant expression. In addition, the 3D model allowed us to design and experimentally obtain mutants that are resistant to auto-degradation and more readily activated.

B3-038P
Molecular cloning and expression of LACTB – a mitochondrial serine protease

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Mitochondria are thought to have originated from a symbiotic relationship between a bacterium able to perform aerobic metabolism ant the ancestor of eukaryotic cells. LACTB is the only mammalian protein showing sequence similarity to bacterial serine proteases and belongs to C class β-lactamases. Mouse LACTB is 551 amino acids long and compromises a predicted mitochondrial import sequence, a short putative transmembrane segment, a β-lactamase homology domain containing the serine protease motif, -SXXK-, and a C-terminal D-transpeptidase domain. The physiological role of mammalian LACTB is unclear. Therefore, the purpose of this research work was to clone the gene of LACTB for expression of LACTB in *E. coli* for further biochemical and cell biological study. The full length lactb gene was cloned into the entry plasmid pENTR/SD/D-TOPO. Expression clones were created performing a recombination reaction between the entry clone and four destination vectors. Expression constructs resulting in N- or C-terminal GST fusion protein and in N- or C-terminal His6-tag fusion protein were transformed into BL21 (DE3) competent cells which are designed for use with bacteriophage T7 promoter based expression systems. When LACTB was expressed as an N-terminal GST fusion protein, full-length LACTB protein was recovered by glutathione-agarose affinity chromatography. Expression of LACTB as a C-terminal GST fusion protein or with either an N- or C-terminal His6-tag resulted in proteolytic degradation of the protein and we were not able to detect full-length LACTB. These results show that the N-terminal GST fragment protects LACTB from proteolytic processing and that LACTB can undergo autoproteolysis, which may be a part of a physiological maturation or activation process.

B3-040P
Design and synthesis of retro-binding peptides active site inhibitors of thrombin

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Thrombin is an important pharmaceutical target for the treatment and prevention arterial and venous thrombosis. Biological active peptides are recognized to have significant therapevtic potential but serious limitations especially for oral dosing. The peptide stereomers could differ when forming productive complexes with an enzyme. Moreover, the replacement of L-amino acid residues forming the hydrolyzed P1–P'1 bond by their enantiomers is known to result in either an uncleavable or a very slowly hydrolyzed analogue. This phenomenon is often used for the synthesis of the peptide's inhibitors stable to the degradation by the enzymes of organism. As the peptides containing D-amino acids, nor are subject to an enzymatic hydrolysis, the purpose of researches was synthesis of a retro – D-analogues of thrombin's substrates constructed from D-amino acids. The di-and tripeptides of the general formula X-D-Arg-D-Phe-OMe [where X = Z, Tos, Ac H, and Z-D-Arg-D-Ala-(D), L-Phe-OMe (OtBu)] were synthesized by conventional methods of peptide synthesis in solution. Special features of their interaction with thrombin are investigated. Their inhibitory action on reaction of splitting of fibrinogen by thrombin and on reaction of a hydrolysis by thrombin βαEE showed, that their inactivating action depends on the substituent on N-end of dipeptides and configuration of phenylalanine in a molecule of tripeptides. The relationship between structure and inhibitory action of the synthesized peptides is discussed. The successful application of D-amino acids for designing of biologically active peptide's analogues as a potential medicinal agent, steady to enzymatic degradation is shown.

B3-041P
Substrate specificity of mannose lectin binding associated serine proteinase 3

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The innate complement system is involved with the neutralization of pathogenic microorganisms. It plays a comparative role to that of the classic immune complement cascade. In the innate complement system, the oligomers of mannose lectins are able to bind to microorganisms. These oligomers have been shown to have mannose lectin binding serine proteinase (MASPs) attached, which once activated lead to the activation of the C3 convertase complex, which finally leads to the formation of the membrane attack complex. There have been three active Masps identified in the human innate immune system- Masp-1, Masp-2 and Masp-3. There is high homology between these three serine proteases especially in the N-terminal regions. They all have two CUB domains, a EGF binding domain, two complement control proteins (CPP) domains and a serine protease domain. The organization of the domains of the Masp proteins are analogous to the domain structure of C1r and C1s. The Masp-1 and Masp-2 have been shown to interact with the Mannose binding lectin and have been implicated in the initiation of the C3 convertase complex. Masp-3 is a splice variant of Masp-1, which has the same N-terminal domain structure of Masp-1 but a different serine protease domain. Little is known about Masp-3 and what its natural targets in the body are. The aim of this work is to determine the natural target for Masp-3 and to determine how the activity of Masp-3 maybe modulated.

B3-042P
Target identification for Spn4, a gene encoding serpin isoforms with alternative reactive site loops

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Serpins are protease inhibitors that present their reactive site loop (RSL) to target proteases, followed by drastic conformational changes that inactivate the protease. The sequence of the RSL of serpins determines the target specificity. The *Drosophila melanogaster* gene Spn4 encodes multiple serpin isoforms each containing an individual RSL, thus enabling the attack of different proteases. Variant Spn4A contains a consensus recognition/cleavage sequence of furin within its RSL and is equipped with a signal peptide and an endoplasmic reticulum (ER) retrieval signal (HDEL). This suggested that the protein resides in the secretory pathway, like furin, a proprotein convertase that activates many cellular proteins and pathogens. Our experiments demonstrate that Spn4A forms SDS-stable complexes with human furin that is inhibited with a second order rate constant of 5.5×10^6/M/s. The RSL of Spn4A is cleaved C-terminally to Arg-Arg-Lys-Arg, in accord with the enzyme's cleavage site. Furthermore, the serpin is retained in the ER of transfected COS7 cells as shown by immunofluorescence staining. A HDEL deletion mutant was detected mainly in the medium of transfected COS7 cells, demonstrating the necessity of the HDEL signal for the observed cellular localization. Further experiments show that furin 1 and 2 of *Drosophila melanogaster* are physiological targets for Spn4A, since secreted forms of both enzymes form stable complexes with the serpin. Together, the results demonstrate that Spn4A is a potent inhibitor of furin that may meet the target at its natural location. Experiments with the other RSL variants show that the Spn4 gene represents a multipurpose weapon that is directed against different families of proteases.

B3-043P
Environmental change caused by substrate binding is the trigger in serine protease catalysis, by increasing of the catalytic histidine Ne pKa

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Formation of the covalent tetrahedral complex (TC) with substrate is the first step of the catalytic process in the active site of serine proteases. His57 (chymotrypsin numbering) plays a role of a general base catalyst, activating the Ser195 nucleophile by abstraction of its proton. It was experimentally observed that the pKa of His57 Ne in TC formed by serine proteases with transition state analog inhibitors is about 5 units higher than the corresponding pKa in the free enzyme. This work demonstrates that the environmental change of the His57 in TC, induced by the substrate binding in the enzyme active site, is the dominant factor in the pKa increase of His57 Ne, and triggers the enzymatic processing of the substrate. These results are based on quantum mechanical modeling of the active site of free chymotrypsin and TC complex of chymotrypsin with trifluoromethyl ketone inhibitor in DFT B3LYP/6-31+G** level of theory. The polar environment of the enzyme active site is accounted for explicitly in the microscopic model. The combined environmental effects of the bulk water solvation and the rest of the protein is implicitly accounted for by our SCRF(VS) continuous solvation approach. The role of local polar effects, such as the oxyanion and the Asp102-His57 hydrogen bond, on the pKa of His57 Ne in TC is analyzed.

B3-044P
Genome-wide analysis of subtilase (subtilisin-like serine protease) genes in microbial genomes

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Subtilisin-like serine proteases (subtilases) are a very diverse family of serine proteases with low sequence homology, often

limited to regions surrounding the Asp, His and Ser catalytic residues. Pattern-searching methods using Hidden Markov Models, based on conserved sequences surrounding the catalytic residues, were used to search for subtilases encoded in >200 bacterial and archaeal genomes, representing 177 species. More than 350 subtilases were found to be encoded in 109 genomes. Subtilases are more commonly found in gram-positive bacteria than in archaea or gram-negative bacteria, and it is more common to have multiple subtilase-encoding genes than a single gene. The majority of the subtilases have a predicted signal peptide for translocation across the cell membrane, and a sub-group of these secreted subtilases are predicted to have a carboxy-terminal cell-envelope anchor, mainly of the LPxTG type for covalent anchoring to peptidoglycan. The genomic context of the subtilase-encoding genes was analyzed to gain insight in putative functions for these proteolytic enzymes. By also taking into account the predicted intracellular or extracellular location of the encoded subtilases, it was possible to predict a function for many subtilases in either nutrition/growth, spore germination, surface protein processing/activation, bacteriocin/toxin processing, or sigma factor activation/regulation.

B3-045P
New coagulant factor isolated from *Bothrops pirajai* venom

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The poisoning by botropics species makes a similar physiologic, one of systemic effects is the blood coagulation for several mechanisms, as direct action on fibrinogen; factor X activation or platelet activation, by toxins of venoms. In the last years were identifies in botropics venoms, serine proteases. This toxins are responsible by coagulant activity with direct action on fibrinogen. Serine proteases are utility for hemostatic system studies and for therapeutics use. Looking for new molecules models is very important to show the mechanism of action and search structural characteristics responsible for its activities. The present work has the objective of purification and characterization of a coagulant factor (CF) from *B. pirajai* venom. The purification was made using a gel filtration, hydrophobic chromatography and an affinity chromatography. The molecular filtration was made in Sephadex G-75 with ammonium bicarbonate buffer (AMBIC) 0.05 M pH 8.1, resulting four fractions (P1–P4), the coagulant fraction was named P1. The P1 fraction was submitted in Phenyl Sepharose chromatography using Triz buffer 10 mM pH 8.6 in a decreasing gradient of NaCl (4; 3; 2; 1; 0.5; 0 M), and to finish the chromatography it was used distilled water, resulting six subfractions (FP1–FP6), the coagulant subfraction was named FP1. The FP1 sub fraction was submitted in Benzamidine Sepharose chromatography and eluted in the solutions: distilled water, obtained the subfraction BFP1, sodium phosphate buffer 20 mM pH 7.8, obtained the subfraction BFP2 and glycine buffer 20 mM pH 3.2, obtained the sub fraction BFP3 that is the CF. The CF displayed one band in SDS-PAGE (11%) showing a pure protein, It has 58 kDa, the minim coagulant dose is 1.75 μg and has action on fibrinogen beta chain.

B3-046P
Deg15 in Arabidopsis thaliana

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The genome of *Arabidopis thaliana* encodes 16 putative proteases from the Deg/HtrA family. This group of ATP-independent serine-proteases was well examined in other organisms, especially *E. coli* and humans, but only limited data is available for members from this protease family in plants. Deg1 and Deg2 have been shown to act as proteases in the chloroplast, but no Deg/HtrA proteases from other compartments have been examined so far. The putative protease Deg15 is predicted to be localized in the peroxisome. We cloned the gene encoding Deg15 (*at1g28320*) in an overexpression vector for heterologous expression in *E. coli*. The tagged protein was purified by affinity chromatography and used to raise polyclonal antibodies. With these antibodies we investigated the intracellular localization of Deg15 and the protein level under various stress conditions in order to evaluate the *in planta* function of this protein.

B3-047P
The effect of site-directed mutagenesis on cold adaptation of VPR; a subtilisin-like serine proteinase from a psychrophilic *Vibrio*-species

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Psychrophilic enzymes have very similar 3D structures as their homologous enzymes from mesophilic and thermophilic organisms. Main characteristics of enzymes from psychrophiles are their high catalytic efficiency (*k*cat/*K*m values) and thermolability. A subtilisin-like serine proteinase from a psychrophilic *Vibrio*-species (VPR) shows these characteristics when compared to homologous enzymes from mesophilic and thermophilic organisms. The VPR gene was cloned, sequenced and expressed in *E. coli* and recently the crystal structure was determined at 1.84 Å resolution [1]. Structural comparisons have been carried out which have led to hypotheses about some of the structural factors which may contribute to cold adaptation of VPR. Some of these hypotheses have been examined using site-directed mutagenesis. The specific residue exchanges were selected with the objective to incorporate stabilizing interactions into the cold adapted enzyme which were deemed to be present in related thermostable homologues. These include incorporation of Pro into loops, a new potential salt-bridge, as well as substitutions aimed at improving packing in the hydrophobic core and decreasing apolar exposed surface. We have also introduced Ser to Ala substitutions at three different locations in the cold-adapted enzyme, but these were the most frequent amino acid exchanges observed in sequence comparisons of the enzyme to those of more thermostable homologues. Here we report on the catalytic and stability characteristics of the selected mutants.

Reference

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B3-048P
Cloning the genes encoding for Kunitz-type proteinase inhibitors group C from potato

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Plant proteinase inhibitors are widely spread in the different plant species being a significant component of a defense system. Somewhere a significant diversity of the proteins related to the same structural family of the inhibitors in the same species may be observed. The family of potato Kunitz-type proteinase inhibitors (PKPIs) exemplifies a group of proteins with the diverse properties and may be divided into three major homology groups: A, B and C. A lot of genes encoding different PKPI-proteins of each group were found in various potato cultivars (*Solanum tuberosum* L.). Inhibition activity of plant invertase, cysteine and serine proteinase was found in proteins subgroup C. A set of gene copies were isolated by PCR from potato cv. Istrinskii genome. DNA sequencing analysis of these resulted in identification of 24 different DNA sequences with a high similarity to potato Kunitz-type inhibitors of group C (PKPI-C). Cluster analysis demonstrated that this clones represented multiple copies of six new genes denoted as PKPI-C1, -C2, -C3, -C4, -C5 and C6. It can be supposed that at least two alleles containing PKPI-C genes are harbored in tetraploid genome of potato. One of new genes, namely PKPI-C5, exhibited 99% identity with known invertase inhibitor cDNA (1423) from cv. Provita. Another PKPI-C6 gene was similar (98% identical residues) with cDNA (p340) from potato cv. Bintje encoding for a putative trypsine inhibitor. Four other new genes demonstrated as much as 89–92% identity with known PKPI-C proteins from other potato cultivars. The N-terminal sequence of the protein encoded by the PKPI-C2 gene was identical to the N-terminal sequence of specific subtilisin inhibitor PKSI isolated from cv. Istrinskii.

B3-049P
Regional distribution of human trypsinogen 4 in human brain determined at mRNA and protein level

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Proteases play an important role in many physiological and pathological processes in the central nervous system such as development, neurite outgrowth, neuronal plasticity and degeneration and cell signaling. A gene coding for such an enzyme might be PRSS3 on chromosome 9 of the human genome. It encodes due to alternative splicing both mesotrypsinogen, which is expressed in pancreas, and trypsinogen 4 whose mRNA has been identified in different human tissues (initially in brain, recently in different epithelial cell lines from prostate, colon and airway). Analysis of the gene PRSS3 predicted two isoforms of the zymogen: Isoform A may have a 72 amino acid, while Isoform B a 28 amino acid N-terminal leader sequence. In order to gain information on the possible role of human trypsinogen 4 we have determined its amount at the mRNA and the protein level as well in 17 selected brain areas using Real-time quantitative PCR and ELISA.The highest transcript levels could be detected in cerebellar cortex, while low amounts were found, e.g. in cerebellar white matter samples. The distribution of the mRNA in different brain areas measured by Real-time PCR is consistent with the protein levels detected with ELISA. The usage of different monoclonal antibodies specific for the 28 amino acid leader sequence and the protease domain allowed the separate detection of the zymogen and the active enzyme. In e.g. the hypothalamus the zymogen is the dominant form, while a significant degree of activation was found in the cerebellar cortex. Our data indicate that the extent of activation varies with different areas. As human trypsinogen 4 is ubiquitous in the brain we conclude that it might play a role in general neurological processes.

B3-050P
Cloning, expression and characterization of human DESC-1, a transmembrane serine protease differentially expressed in head and neck squamous cell carcinoma

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Serine proteases are enzyme involved in the maintenance of the cell homeostasis. Thus, this type of enzymes must be extremely regulated and it has been highly reported that serine proteases are involved in the growth and expansion of different cancers. In this regard, the Type II Transmembrane Serine Proteases (TTSPs) constitute a subfamily of membrane anchored serine proteases that are ideally positioned to carry out different interactions with other cell surface or extracellular proteins. Among them, TMPRSS2 and TMPRSS3 proteins have been reported to be overexpressed in most prostate and ovarian cancers respectively, matriptase/MT-SP1 is expressed in a wide variety of benign and malignant tumors and hepsin is overexpressed in ovarian and renal cancers. DESC-1 is a TTSP member found differentially expressed in squamous cell carcinoma (Differentially Expressed in Squamous Cell Carcinoma Gene 1) and differentially from other TTSPs, its expression is found to be reduced in tumor tissues respecting to the normal tissue at RNA level in Head and Neck Squamous Cell Carcinoma (HNSCC), what suggests a possible tumor protective function for DESC-1. In order to shed light about the role of DESC-1 in these processes, we have carried out the molecular cloning of the human full-length cDNA and expression of the recombinant protein to delineate the implication of this protease in HNSCC.

B3-051P
Engineering of GFP for the screening of serine protease inhibitors

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Site specific proteolysis has been an attractive target for the development of antiviral therapies based on selective viral inhibitors. It has been previously demonstrated that reporter proteins like beta-galactosidase could be very useful for the high-throughput screening of HIV-1 protease inhibitors through the display of an accessible protease target site on the enzyme surface. In this work, by using structural analysis, we have engineered the GFP protein from jellyfish *Aequorea victoria* to accommodate in its surface the HCV virus NS5A-5B protease cleavage site EDVVCCSMSYTWTG, in a manner that proper proteolysis results in a fluorescent activity decrease. The three resulting GFP constructions, carrying the protease cleavage site in positions 23–24, 102–103 and 172–173, were soluble expressed in *Escherchia coli*. Moreover, the HCV NS4 cofactor residues 21–34 fused in frame via a short linker to the amino terminus of the HCV NS3 protease domain (residues 2–181) were also expressed in *E. coli* and under 1mM IPTG induction, at least 60% of soluble protein was recovered and further purificated by an histidin tag. The analysis of GFP proteolysis in front of HCV recombinant protease were performed either with bacteria crude extracts and purificated proteins. The results presented here indicated that proper solvent exposure of target sites on GFP carrier protein may be a critical factor for protease cleavage and for the observation of fluorescence activity variance, being an aspect of absolute relevance for further design and implementation of newer analytical tests.

B3-052P
Activities of serine proteinases and serine proteinase inhibitors in idiophasic cultures of white-rot basidiomycete *Abortiporus biennis* under oxidative stress conditions

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Various kinds of stressors cause the group of metabolic changes defined as the general stress response, initiated by some intracellular signals, such as production of abnormal or denaturated proteins, enhanced generation of reactive oxygen species and others. Proteolytic enzymes quickly modify proteins and as a consequence can regulate cellular metabolism. Although the stress defense mechanisms have been very often described in the recent literature, in very few works were estimated stress response abilities of white-rot basidiomycetes, which produce two kinds of very important ligninolytic enzymes – laccase and peroxidases. Our previous results showed that the addition of menadione to *Abortiporus biennis* idiophasic cultures caused the significant increase of the extracellular laccase activity in comparison to the control. The aim of this study was to determine activities of serine proteinases and natural serine proteinase inhibitors in idiophasic cultures of basidiomycete *A. biennis* grown under menadione-mediated oxidative stress conditions. We investigated the changes of intracellular serine proteinases activities in the presence and absence of ATP, using hemoglobin and fluorogenic substrates. The level of natural serine proteinase inhibitors in mycelia was also measured. A fungal inhibitor of trypsin was partially purified and used to *in vitro* experiments. An interesting correlations between serine proteinases, serine proteinase inhibitors and laccase activities in prooxidant treated cultures were also observed. It can suggest that the proteolytic modifications under oxidative stress conditions can act as a regulation way of laccase activity. Serine proteinases, inhibitory and laccase activities were additionally analyzed by native PAGE.

B4–Regulatory Proteases

B4-001
Calpain and connectin/titin in health and disease of skeletal muscle.

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Calpain is a Ca^{2+}-regulated cytosolic cysteine protease, functioning as a "modulator protease", i.e. regulating/modifying functions/activities of substrates by limited proteolysis to modulate cellular functions. Human has 14 calpain genes and potential substrates extend to various cytosolic proteins such as kinases, transcription factors, cytoskeletal and ER proteins. In skeletal muscles, expression of p94 (also called calpain 3) predominates, playing an indispensable role for muscle functions in cooperation with ubiquitously expressed conventional calpains. For, a defect of p94 proteolytic activity originated from gene mutations causes muscular dystrophy. p94 localizes in myofibrils binding to connectin/titin, a gigantic elastic muscle protein connecting the Z- and M-lines of sarcomere, the repetitive unit of myofibril, with a single molecule. In *mdm* (muscular dystrophy with myositis) mice, connectin/titin with a small deletion caused by natural mutation of the connectin/titin gene is expressed, resulting in severe muscular dystrophy phenotypes such as body weight less than a half of that of wild type, severely affected limb muscles with impaired walking ability and only 2–3 months of life time. The deletion in the *mdm* allele of the connectin/titin gene overlaps one of the binding sites of p94 in the N2-line, another electron-microscopically visible line between the Z- and M-lines of sarcomere. The *mdm* phenotypes clearly indicate that connectin/titin or p94 or both are essential for proper muscle functions. To elucidate physiological roles of connectin/titin and p94, we analyzed *mdm* mice in relation to calpain system. As a result, MARPs (muscle ankyrin repeat proteins) were shown to be up-regulated in *mdm* muscle. MARPs bind to the N2- and Z-line regions of connectin/titin and function as transcriptional

regulators translocating into the nuclei. CARP (cardiac ankyrin repeat protein), one of MARPs, binding site in the N2-line region is proximate to the p94 binding site, thus suggesting interactions of both molecules. Possible signal transduction systems to modulate muscle functions revealed by the analyses will be discussed based on the results.

B4-002
Inhibition and activation of calpain by its disordered endogenous inhibitor, calpastatin

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Calpains are a family of intracellular calcium-activated cysteine proteinases, implicated in the regulation of key cellular processes, such as cell division and programmed cell death. Their activity is under tight control by an intracellular protein inhibitor, calpastatin, an intrinsically unstructured protein that contains four equivalent inhibitory domains. Each of these comprise three conserved subdomains, of which subdmomains A and C anchor the inhibitor in a calcium-dependent manner, whereas subdomain B binds at the active site and inhibits the enzyme. In this work it is shown that the consequence of this mode of binding is that isolated A and C peptides promote calcium binding to calpain and thus activate the enzyme. This activation is manifest in the sensitization to calcium ion: the calcium required for half-maximal activity is lowered from 4.3 to 2.4 μM for μ-calpain and 250 to 140 μM for m-calpain. In the physiologically significant sub-micromolar and low micromolar calcium concentration range this sensitization leads to a more than tenfold activation, which is of potential physiological importance as isolated calpain requires high calcium concentrations never realized *in vivo*. Here we suggest calpastatin is degraded *in vivo* in a way that generates the activator peptides. Due to the structural disorder of calpastatin, this unprecedented mode of action raises intriguing questions with respect to the generality of this ambivalent behavior. To address this issue, we have collected extreme cases, when the same protein elicits opposing, inhibitory and activatory, responses within the same molecular setting: structural predictions show that these proteins are largely disordered. As a conclusion, the possible general implications of this finding are discussed.

B4-003
Meprin metalloproteases in inflammation and cancer

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Meprins are oligomeric, brush border membrane or secreted zinc proteases that have unique and complex structures. They are composed of multidomain, highly glycosylated evolutionarily-related α and β subunits that form disulfide-linked homo- or heterooligomeric dimers. The homooligomeric form of meprin A forms very high molecular mass multimers of 1 000 000–6 000 000 Da, among the largest extracellular proteolytic complexes known. Meprins cleave cytokines, growth factors, bioactive peptides and extracellular matrix proteins, important compounds in inflammatory intestinal disease and in cancer metastases. To investigate the role of meprins in intestinal immune responses, inflammation was induced in mice by oral administration of dextran sulfate sodium (DSS). The results showed that wild-type mice (C57Bl/6 × 129) had a more severe reaction to DSS than meprin β null mice on the same genetic background, as determined by body weight loss, intestinal bleeding and mortality. This implies that the presence of meprin β increases host damage caused by DSS and that meprin β plays an active role in intestinal pathophysiology. Meprins are also expressed in colon cancer cells (e.g. SW480, SW620, and CaCo-2). Expression of meprin α appears to increase with increasing metastatic potential. In addition, meprin α is highly expressed in the human liver hepatoblastoma cell line HepG2 and abundantly secreted into culture media. Examination of human tumor samples showed that meprin α is expressed in primary colon tumors and in tumors that have metastasized to the liver. This indicates that meprin α expression in gastrointestinal tumor cells contributes to the progression of the disease.

B4-004
Biochemical pathways mediating necrotic cell death and neurodegeneration in *Caenorhabditis elegans*

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Necrotic cell death plays a central role in devastating human pathologies such as stroke and neurodegenerative diseases. Elucidation of the molecular events that transpire during necrotic cell death in simple animal models should provide insights into the basic biology of inappropriate neuronal death, and facilitate the characterization of mechanisms underlying degeneration in numerous human disorders. Various cellular insults, including hyperactivation of ion channels, expression of human beta-amyloid protein implicated in Alzheimer's disease, constitutive activation of certain G proteins, hypoxia and possibly the ageing process, can trigger a degenerative, necrotic cell death in the nematode *Caenorhabditis elegans*. We are genetically and molecularly deciphering the *C. elegans* necrotic death program. We have isolated mutations in several distinct genetic loci that bock degenerative cell death initiated by various genetic and environmental insults. By characterizing such suppressors, we have discovered that neuronal degeneration inflicted by various genetic lesions in *C. elegans*, requires the activity of specific calcium-regulated calpain proteases and acidic pH-dependent aspartyl proteases. Although, it is believed that these proteases become activated under conditions that inflict necrotic cell death, the factors that govern the erroneous activation of such—otherwise benign—enzymes are largely unknown. We identified novel factors that modulate cellular pH homeostasis, which are required for necrosis and showed that targeting these factors effectively protects from necrotic cell death in *C. elegans*. Our findings demonstrate that two distinct classes of proteases are involved in necrotic cell death and suggest that perturbation of intracellular calcium levels may initiate neuronal degeneration by compromising pH homeostasis and deregulating proteolysis.

B4-005
Search for regulatory proteins which are controlled by proteolysis in *Escherichia coli* based on microarray analysis

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The impact of controlled proteolysis on regulatory events in prokaryotes is increasingly recognized over the last decade. As in eukaryotic cells, proteolysis is more than just a garbage disposal

but has been found to be implicated in the regulation of many vital functions of the bacterial cells, like cell cycle, stress responses and development (Hengge R and Bukau B. *Mol Microbiol* 2003). Conditional degradation of regulators shows a high potential of integrating a great variety of signals as is well studied for the degradation of Sigma S. This Sigma subunit of the RNA polymerase, which triggers the general stress response in *Escherichia coli* is digested rapidly by the ClpXP protease in association with the phosphorylated response regulator RssB under non-stress conditions (Stuedemann A. *EMBO* 2004). Several other regulatory proteins have been found to be subjected to proteolysis, as LexA, a regulator of the SOS response. Also Lon protease is involved for example in the degradation of RcsA, a regulator of the capsule biosynthesis and of SulA, a cell division inhibitor (as a review: Hengge-Aronis R, Jenal U. *Curr Opin Microbiol* 2003). In order to find other regulatory processes in which proteolysis plays a role we pursued a global approach using the microarray technique. In mutants lacking functional ClpP or Lon proteases or either one of the Clp recognition factors ClpA and ClpX, we searched for genes, which are differentially transcribed compared to the wildtype. We found some interesting groups of genes belonging to common regulons governed by known regulators - candidates for Clp or Lon mediated proteolysis. After confirmation of these results through lacZ fusion studies of representative genes of these regulons, these regulators are presently examined in *in vivo* degradation studies using immunodetection methods.

B4-006
β-propellers in enzyme catalysis and regulation

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A distinct group of serine peptidases cannot hydrolyze proteins, but can readily cleave peptides that are up to about 30 amino acid residues long. The representative member of the family, prolyl oligopeptidase is implicated in a variety of disorders of the central nervous system. The enzyme consists of a peptidase domain with an α/β-hydrolase fold and its catalytic triad is covered by the central tunnel of a seven-bladed β-propeller. This domain makes the enzyme an oligopeptidase by excluding large structured peptides from the active site. In most propeller domains the circular structure is ''velcroed'' together in a mixed blade, where both amino and carboxy terminus are involved to form a four stranded antiparallel β-sheet. Non-velcroed or ''open topology'' propellers are rare, and prolyl oligopeptidase was the first protein structure exhibiting a domain of this nature. The apparently rigid crystal structure does not explain how the substrate can approach the catalytic groups. Two possibilities of substrate access were investigated: either blades 1 and 7 of the propeller domain move apart or the peptidase and/or propeller domains move to create an entry site at the domain interface. Engineering disulfide bridges to the expected oscillating structures prevented such movements, which destroyed the catalytic activity and precluded substrate binding. This indicated that concerted movements of the propeller and the peptidase domains are essential for the enzyme action.

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And references therein.

B4-007P
Biochemical characterization of *Thermoplasma volcanium* recombinant 20S proteasome and its regulatory subunit

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Proteasome associated energy dependent proteolysis is not only involved in rapid turnover of specific proteins that could be important during periods of stress, but also engaged in the turnover of the short-lived proteins that regulate a variety of cellular processes in both procaryotic and eucaryotic cell. The universal distribution of proteasome homologs in archaeal genome provide insight into the vital role of archaeal proteasomes. 20S catalytic core of archaeal proteasomes in combination with various AAA ATPases and membrane associated Lon proteases may play role in stress response or turnover of the regulatory proteins. However, little is known about the potential physiological roles of archaeal proteasomes. This study presents the data on biochemical and biophysical features of recombinant 20S proteasome of a thermoacidophilic archaeon *Thermoplasma volcanium* (Tpv). PCR was performed to amplify DNA fragments containing Tpv genes encoding the α - and β-subunits of the proteasome from Tpv genomic DNA. The amplified α-gene (TpvA) and β-gene (TpvB) together with their upstream sequences were separately cloned and then combined in pUC18 vector. The resulting recombinant pUC-SK-βα plasmid was used for heterologous production of *in vivo* assembled 20S proteasome in *E. coli*. The recombinant proteasome was purified by combination of ammonium sulfate precipitation, gel filtration chromatography (Sepharyl S-300) and ion-exchange chromatography (Q Sepharose). Molecular masses of purified protein subunits were estimated as 23.71 kDa (β-subunit) and 21.13 kDa (α-subunit). Substantial post-glutamyl peptide hydrolyzing activity and chymotrysin–like activity were detected as associated with recombinant proteasome. Maximum chymotrypsin-like activity was measured at 85 °C and pH 8.5.

B4-008P
Crystallographic studies of the GTP-dependent transcriptional regulator CodY from *Bacillus subtilis*.

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CodY is a GTP dependent transcriptional regulator of early stationary phase and sporulation genes in *Bacillus subtilis*. It is activated by GTP, during rapid cell growth it represses several genes whose products allow adaptation to nutrient depletion. When the cells pass from rapid growth to stationary phase, the intracellular concentration of GTP drops thus releasing the repressed genes. Cod Y is a 259-residue polypeptide containing a helix-turn-helix motif for binding to DNA. It also has motifs common with small GTPases, but CodY has a much lower affinity for GTP. Crystals of the full-length CodY have been grown in the presence and absence of GTP from sodium citrate buffered solutions using lithium sulphate as a precipitant and diffraction data have been collected to 3.5 Å resolution. Attempts to solve the structure using anomalous data from the SeMet derivative crystals of CodY have been hampered by the large number (~70) of methionines in the asymmetric unit and difficulties in reproducibility of

diffracting crystals. Therefore we used limited proteolysis and mass-spectrometry analysis to identify the domain boundaries of the protein and were able to determine the sequence of two principal proteolytic fragments corresponding to the N- and C-terminal domains of CodY. These individual domains which were successfully cloned in *Escherichia coli*, overexpressed as His-tagged proteins, isolated and purified. Both domains have been crystallized. The crystals of the N-terminal domain grow from Bis-Tris buffered solutions at pH 6.5 containing polyethylene glycol and calcium acetate. The crystals of the C-terminal domain were obtained using ammonium sulphate as a precipitant. The crystals of N-terminus domain diffract to at least 2.3 Å and crystals of C-terminus domain – to 3.2 Å using an in-house diffractometer with a MAR research image-plate as a detector. Progress towards the determination of CodY structure will be presented.

B4-009P
Signaling pathways implicated in oncostatin M-induced aggrecanase-1 and matrix metalloproteinase-13 expression in human articular chondrocytes

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Levels of a major pleiotropic, interleukin-6 family cytokine, oncostatin M (OSM) are increased in the synovial fluid of patients with rheumatoid arthritis where it contributes to the catabolism of cartilage by inducing collagen degrading matrix metalloproteinase, MMP-13 and aggrecan cleaving, ADAMTS-4/aggrecanase-1. Poorly understood mechanisms of OSM stimulated ADAMTS-4 and MMP-13 increases were investigated in human chondrocytes from arthritic patients. Pre-treatment of human femoral head chondrocytes with extracellular signal-regulated kinases (ERK1/2)-MAPK pathway inhibitors, U0126, resulted in suppression of ADAMTS-4 mRNA and MMP-13 induction by OSM. Janus kinase (JAK) inhibitor 3 and signal transducer and activator of transcription (STAT3) phosphorylation inhibitor, parthenolide, also reduced OSM-induced ADAMTS-4 and MMP-13 gene expression. Parthenolide prevented STAT3 DNA binding activity of nuclear extracts from human SW1353 chondrosarcoma cells. Additionally, OSM-induced ADAMTS-4 mRNA and MMP-13 expression was down regulated by phosphoinositide 3-kinase (PI3K) (LY294002) and AKT/PKB (NI-71-101) inhibitors. Furthermore, JAK3 inhibition time-dependently down regulated AKT but not ERK1/2 phosphorylation suggesting that AKT is a downstream target of JAK3. These results suggest that OSM-stimulated ADAMTS-4 and MMP-13 expression is mediated by ERK1/2, JAK3/STAT3 and PI3K/Akt and by cross talk among these pathways. The inhibitors of these cascades could potentially block OSM-evoked inflammation and degeneration of cartilage by ADAMTS-4 and MMP-13.

B4-010P
Proteolytic release of membrane-anchored proteins in *Listeria Monocytogenes* and its role in the virulence

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The gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular parasite. Interactions of *L. monocytogenes* with the host cell are provided by a number of secreted and cell surface proteins. One of the most important virulence factors, actin-polymerizing protein ActA, is surface attached via the hydrophobic C-tailed membrane anchor. Despite, the membrane anchor ActA was found in comparable amounts both on the cell surface and in the culture supernatant. The aim of the work was to investigate the mechanism of ActA release and the role of this process in *L. monocytogenes* virulence. MALDI-TOF MS analysis of trypsin released ActA suggested releasing due to proteolytic cleavage between histidine and threonine residues in the close vicinity of the membrane anchor predicted by the HTMM analysis. The substitution of histidine with proline prevented ActA release into the culture supernatant, although did not disturb its surface presentation. In silico analysis of eight other *L. monocytogenes* membrane-anchored surface proteins suggested the role for asparagine and threonine residues in specific proteolysis. The prediction was experimentally tested by substitution of the residues with alanine. The *L. monocytogenes* spontaneous mutant strain, unable to release membrane-anchored proteins into the culture supernatant, was isolated. The mutation was mapped outside the actA gene and presumably affected the corresponding peptidase. The mutation impaired the invasion of *L. monocytogenes* into the human epithelial-like HeLa cells that suggested the effect of the released proteins on signaling events that result in induced phagocytosis of the pathogen by normally non-phagocytic cells.

B4-011P
Serotonin–induced ERK phosphorylation involves ADAM-17/TACE activation in mesangial cells

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We have shown recently that serotonin (5-HT) causes phosphorylation of extracellulary regulated kinases 1 and 2 (ERK 1/2) through epidermal growth factor receptor (EGFR) transactivation in mesangial cells. The mechanism involved shedding of heparin binding EGF (HB-EGF) and was metalloproteinase dependent. HB-EGF co-precipitated with ADAM-17/TACE, but not with ADAM-9, -10, -12 or -15, suggesting that ADAM 17/TACE is the metalloenzyme that processes HB-EGF in human mesangial cells. To confirm a role of ADAM-17/TACE, we used fluorogenic peptide substrates. Employing Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH2 (which is an excellent substrate for ADAM-17), we observed $\sim$ 35% increase in enzymatic activity in the media of 1 mM 5-HT-treated cells compared to untreated cells. However, we did not see any increase in the fluorescence when we used "CatE1", an ADAM substrate, which does not recognize ADAM-17. To further support a role for ADAM-17/TACE, we designed silencing RNAs against the enzyme, which were introduced into the mesangial cells using lentiviral infection. Successful silencing was confirmed by Western blotting 4 days after infection. Control and TACE silenced human mesangial cells were stimulated with 2–10 μM of serotonin for 5 min, and ERK activation was assessed by Western blotting. The 5-HT-induced ERK phosphorylation was completely attenuated in TACE silenced cells compared to controls confirming that TACE is the metalloenzyme that processes HB-EGF during 5-HT_{2A} receptor and EGFR crosstalk.

B4-012P
Formation and degradation of angiotensin II by human keratinocytes in culture

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Angiotensin II (Ang II) has been proposed to act as a regulatory peptide in the epidermal layer of human skin. While the expression of receptors and peptide precursors have been demonstrated in epidermis, the formation of Ang II and its inactivation have not been studied in detail. Thus we have established a model system with cultured keratinocytes to examine the metabolism of Ang I, II and related peptides by intact epidermal cells. Cultures were incubated with peptides in a minimal medium, which sustained cell viability for at least 24 h and the metabolism of peptides was monitored by chromatography (RP-HPLC). With Ang I as peptide substrate five major products were detected in keratinocyte culture media after 12 h incubation. A half-life of about 9 h was estimated for Ang I and the slow degradation supports results of earlier studies revealing low activities of exopeptidases in a microsomal fraction from keratinocytes as compared to fibroblasts. The degradation of Ang I was not affected by inhibitors of alanyl aminopeptidase, peptidyl dipeptidase A and neprilysin. Since a peptide product formed from Ang I in keratinocyte cultures resembled Ang II in HPLC analysis, the activity of peptidyl dipeptidase A in these cells was assayed with Hip-His-Leu and the presence of the peptidase was confirmed by its sensitivity to captopril. Further experiments showed that Ang II, III and related peptides were degraded in keratinocyte cultures with rates similar to Ang I and these reactions interfered severely with the formation of Ang II. Immunohistochemical studies showed a strong positive staining for neprilysin and alanyl aminopeptidase in the dermal layer of human skin and at the epidermal-dermal junction confirming the results obtained with the cell cultures.

B4-013P
Soluble angiotensin converting enzyme-2 present in human plasma and urine

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Angiotensin-converting enzyme (ACE) is a zinc metallopeptidase critical for the generation of the vasoconstrictor peptide angiotensin II. A homologue of ACE, ACE-2, has recently been identified, which appears to play a counter-regulatory role to ACE by inactivating angiotensin II. Like ACE, ACE-2 is a Type I membrane protein with its active site contained within the extracellular domain. The expression of ACE2 protein is normally low and restricted primarily to endothelial cells of the heart and kidney, kidney epithelium and testis. Recent evidence from ourselves and others indicates that ACE2 is significantly upregulated in a number of pathologies, such as myocardial infarction, renal disease and hepatitis C-induced cirrhosis. Given that ACE can be proteolytically released from the cell surface in culture, ACE2 may likewise be shed into plasma or urine. Detection of elevated levels of ACE2 in plasma and urine may be a useful biomarker for the diagnosis of hepatic, renal and vascular disease. Using a specific quenched fluorescent substrate, we have detected ACE2 activity in human urine. In contrast, ACE2 activity could not be detected in human plasma; interestingly, however, we noted that plasma markedly inhibited the activity of recombinant ACE2, thus compromising the possibility of measuring plasma enzyme activity. We are in the process of purifying this inhibitor, which preliminary results suggest is small and hydrophilic. We are also currently optimizing methods for its removal from plasma samples, thus allowing detection of low levels of soluble ACE2 activity in normal human plasma. The identification of a potential endogenous inhibitor of ACE2, the first for this family of metallopeptidases, could have significant consequences for ACE2 function *in vivo* and the regulation of angiotensin peptides. Future studies will examine whether plasma or urinary levels of ACE2 are elevated in cardiovascular, renal or liver disease.

B4-014P
Molecular determinants of proteolytic processing of non-structural polyprotein of Semliki forest virus.

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Semliki forest virus (SFV) is a positive-stranded RNA virus. The replication of SFV is performed by the RNA-dependent RNA replicase complex (RC) and regulated by proteolytic processing. During the course of the infection template preference of RC changes from RNA plus-strand to minus-strand. It has been known for several years that this preference switch is due to the proteolytic processing of SFV non-structural polyprotein p1234, mediated by viral cysteine protease located in the carboxy-terminal domain of the nsP2 protein. Tight temporal regulation of this template specificity switch is crucial for the viral replication, but, nevertheless, its mechanism remains unsolved. Therefore, the mapping of the essential molecular determinants of the site-specific cleavage consensuses may provide necessary information, concerning the cleavage regulation as well as regulation of the RNA replication. The results of our studies indicate that as little as 5 amino acid residues from the C terminus of nsP3 protein determine the specificity of the proteolytic cleavage of the nsP3/nsP4 junction. At the same time sequences laying downstream of the cleavage point (in nsP4 region) have only minor effect on the cleavage efficiency. The exact region required for the cleavage of nsP2/nsP3 junction is yet not known but the sequences, required from C-terminal part of nsP2 protein, are likely short as well. In contrast, sequence lying within 80–240 N-terminal amino acid residues of nsP3 is vital for cleavage of the nsP2/nsP3 junction. This region may represent the cofactor of the nsP2 protease that activates processing at the nsP2/nsP3 cleavage site. Thus, as the result of current research, a principally new function – regulation of the proteolytic processing and RNA replication – was mapped to the conserved N-terminal region of the nsP3. This finding significantly improves our understanding about the role of nsP3, which was enigmatic till now, in the virus life cycle.

B4-015P
Functional properties of p94/calpain3 and connectin/titin in *mdm* mouse skeletal muscle

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p94/calpain 3 is the skeletal-muscle-specific calpain and is considered to be a modulator protease in various cellular processes. A defect in the p94 gene causes limb-girdle muscular dystrophy type 2A (LGMD2A), suggesting that p94 functions are indispensable for proper muscle functions. In sarcomeres, p94 localizes at Z-, N2- and M line regions. Although the binding partner for p94 at Z-line has not been identified yet, N2- and M-line localization of p94 are considered dependent on its interaction with the N2A and M-line regions of connectin/titin, respectively. Connectin is a gigantic sarcomeric protein playing an important role as a molecular template for sarcomeric organization, an elastic element generating passive tension, a platform for various protein ligands, etc. In this study, we focused on the molecular components associated with the N2A region of connectin/titin to extend our understanding on p94. Intriguingly, a recessive mutation in the mouse connectin gene, *mdm* (muscular dystrophy with myositis), causes muscular dystrophy. There are two remarkable phenotypes consequential to *mdm* mutation. First, the *mdm* mutation abolishes p94 binding activity of connectin N2A fragment. Second, in skeletal muscle from mice homozygous for *mdm* mutation, upregulation of cardiac ankyrin repeat protein (CARP) is observed. CARP also binds to N2A connectin at the N-terminal proximity of the region mutated by *mdm*. The effect of *mdm* mutation on p94 activity and the properties of N2A connectin as well as CARP were analyzed using both animal model and cell culture systems.

B4-016P
Sequencing and functional identification of conditional lethal mutants of Semliki forest virus

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Semliki forest virus (SFV) is well known model virus, which has been studied for decades. The main topic of this research was characterization and analysis of SFV replication machinery using approach based on use conditional-lethal mutants of viruses. The direct aim of the present study was to sequence and functionally characterize a panel of independent SFV temperature sensitive mutants. From all putative ts-mutations, identified in this study, two were mapped to nsP1 protein, four were mapped to nsP2 protein and one was founded in nsP4 region. Number of assays were used to verify phenotypic effects of revealed mutations: titration of virus stocks at different temperatures, leak yield experiments, analysis of viral RNA synthesis and viral polyprotein processing at different temperatures. NsP2 mutants had clear viral protease defect and accumulated non-cleaved polyproteins on different stages. Besides all, biotechnological branch of our research is already developing. It includes improving of existing SFV based expression vector system by use of ts-mutations for the temperature regulation of foreign gene expression in mammalian cells.

B4-017P
Dipeptidyl peptidase IV activity and/or structure homologues (DASH) in brain tumors

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Pathogenesis of many diseases, including cancer, often involves improper proteolytic post-translational modification of biologically active peptides. Association of dysregulated expression pattern of novel group of "Dipeptidyl peptidase (DPP)-IV Activity and/or Structure Homologues" (DASH) with cancer development and progression has been suggested by several authors, including us [1]. DPP-IV enzymatic action as a common attribute of most of DASH members modifies signaling potential of their substrates, biologically active peptides, not only quantitatively, but due to the changes in their receptor preferences also qualitatively. In this study, we have investigated expression (by real time RT-PCR and immunohistochemistry) and enzymatic activity (by biochemical assays and enzyme histochemistry) of plasma membrane localized DASH members, in particular DPP-IV, fibroblast activation protein-alpha (FAP) and attractin in human gliomas. It was revealed that varying quantities of DPP-IV, FAP and attractin mRNAs and proteins were coexpressed in the studied tumors. The majority of DPP-IV-like activity in the glioma tissue could be attributed to the canonical DPP-IV. This activity, assayed biochemically and expressed per mg of protein, was increased in high grade gliomas. Inhibition studies suggested lack of enzymatically active attractin in the examined glioma tissues. The results of our pilot study demonstrate for the first time that both enzymatically active and inactive DASH molecules are coexpressed in gliomas and suggest prevailing association of increased DPP-IV activity with high grade tumors.

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B4-018P
Importance of VEGF-C processing by the proprotein convertases in Zebrafish fin regeneration

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VEGF-C is involved in the neovascularization processes, steps essential for wound healing, cancer progression and many other physiological functions. Zebrafish VEGF-C processing and

activation occurs within the specific dibasic motif HSIIRRSL, suggesting the involvement of the proprotein convertases (PCs) in these process. This family of endoproteases are responsible for the activation of a large variety of regulatory proteins by cleavage at multi-basic recognition sites exhibiting the general motif (K/R)-(X)n-(K/R)(n = 0, 2, 4 or 6). Cotransfection of the furin-deficient colon carcinoma cell line LoVo with proVEGF-C and different PC members revealed that furin, PC5 and PC7 are VEGF-C convertases. The processing of proVEGF-C is blocked by the inhibitory prosegments of furin, PC5 and PACE4, as well as by furin-motif variants of alpha2-macroglobulin and alpha1-antitrypsin. Accordingly, mutation of the VEGF-C PC-site (HSIIRRSL to HSIISSSL) inhibited proVEGF-C processing. Following Zebrafish caudal fin amputation, the injection of control vector or vector containing wild VEGF-C did not affect fin regeneration. In contrast, injection of muted VEGF-C (proVEGF-C) inhibited fin regeneration. These data highlight the importance of VEGF-C processing in Zebrafish fin regeneration and suggest that Zebrafish can be used as a simple and useful model for studying the role of protein maturation by the PCs in physiological processes.

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Substrate specificity of thimet oligopeptidase (EC3.4.24.15) depends on loop flexibility

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Thimet oligopeptidase (TOP) hydrolyzes a variety of bioactive peptides and is implicated in the regulation of neurological and other physiological processes. TOP is composed of two ''clam-shell'' domains, with the substrate-binding pocket and catalytic site lying between these domains. It is speculated that conformational changes in loops and coil regions connecting the domains lead to changes in substrate specificity. The loop region (residues 599–611) is close enough to the active site to interact with even the smallest substrate. It contains three glycine residues and is expected to be quite flexible. In an effort to trap intermediate conformations of the loop, we have replaced Gly 599, 603, or 604 with Ala and have compared the activities of the three resulting protein constructs towards two quenched fluorescent substrates. All three enzymes had lower activity than wild type towards a bradykinin analog, with G599A, the most active of the mutants, possessing 1/3 wild-type activity. However, utilizing a smaller substrate, G603A was the most active, surpassing even wild type (~fivefold increase in activity). G604A had little activity towards either substrate. These results are consistent with data that revealed increases in activity towards the larger substrate, when the enzyme is partially denatured and presumably, more flexible and with increased accessibility of the binding loop to proteolytic enzymes, when partially denatured.

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