FEBS ICGEB 2022 Advanced Course

PROTEOLYSIS

at the interface between health and disease

17-21 September | Bled, Slovenia

International Centre for Genetic Engineering and Biotechnology

BOOK OF ABSTRACTS

Scientific and organizing committee:

Maja Orehek, Andreja Kozak, Robert Vidmar and Boris Turk

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IUBMB Keynote opening lecture
(chair: Boris Turk)

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21:00 – 23:00 Get together, networking, chill and chat (HOTEL LOVEC)

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(chair: Klaudia Brix)

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10:30 – 10:45 **Lisa Douglas**: Highly selective furin inhibition as a therapeutic approach for chronic obstructive pulmonary disease

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10:30 – 10:45 **Robert Vidmar**: Emerging role of legumain in innate immune response – potential link to anti-inflammatory effects

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12:00 – 12:30 **Marcin Poreba**: Remarkable potential of unnatural amino acids for the development of protease-responsive prodrugs

12:30 – 12:45 **Andrea Smith**: New protein reactive centers against aspartic proteases

12:45 – 14:45 LUNCH BREAK (HOTEL LOVEC)

14:45 – 20:00 Free afternoon

20:00 – 24:00 GALA DINNER, POSTER AWARD ANNOUNCEMENT and Conference Closing (HOTEL LOVEC)

DAY 5 - Wednesday, September 21st 2022

Hotel check-out & Departure

LIST OF LECTURES

Evasion of cellular anti-viral defences deciphered by TAILS Analysis of SARS CoV-2CLpro substrates in COVID-19

C. Overall[I]

[I]*University of British Columbia, Vancouver, Canada*

We delineate the interconnected human protein substrate landscape of 3CLpro using TAILS of human lung and kidney cells treated or not with interferons, supported by analyses of SARS-CoV-2-infected human lung cells. Over 100 substrate discoveries were identified and further validated by MALDI-TOF analysis of synthetic peptide cleavage kinetics for each cleavage site in >100 cut sites. Molecular docking simulations of 3CLpro engaging substrates confirmed the ~10% of validated sites were noncanonical that diverge from SARS-CoV to guide substrate and inhibitor drug specificity. PPI analysis shows that cleavage by 3CLpro of the interactors of essential effector proteins, effectively strands these from their binding partners so amplifing the consequences of proteolysis throughout the cell by protein complex disassembly. Using recombinant protein digestion, Edman degradation, and digestion of normal human bronchial epithelial cell from 5 subjects we further confirmed substrate cleavages. We show that 3CLpro targets multiple proteins in the Hippo pathway, including inactivation of MAP4K5 and YAP, as well as effectors of transcription, mRNA processing and translation. We discovered that SARS CoV-2 Spike protein binds galectin-8, cleavage of which disengages CALCOCO2/NDP52 and decouples protective anti-viral-autophagy. Indeed, unlike healthy lung, in post-mortem COVID-19 human lung samples obtained from the Tissue Human Protein Atlas, NDP-52 rarely colocalizes with galectin-8. Thus great mechanistic insight to novel mechanisms of evasion of antiviral host cell immunity, hijacking of the host mRNA binding and translation apparatus, disruptions in cell shape and syncytium formation was enabled by rationale start points identified in the 3CLpro substrate degradome. The Atlas of CoV-2 substrates establishes a foundational resource to accelerate further exploration of SARS-CoV-2 pathology in the COVID-19 cellular coup d'etat.

Pablos et al. https://doi.org/10.1016/j.celrep.2021.109892

MMP-9 as a key player in development of epilepsy

L. Kaczmarek[I]

[I]*Nencki Institute, Warsaw, Poland*

Matrix metalloproteinase 9 (MMP-9) is an extracellularly/pericellularly operating protease that regulates numerous cell activities, such as cell differentiation, cell migration, cytokine release, survival, apoptosis, inflammation, and cell-cell contacts. In the brain, MMP-9 is expressed and released by neurons and glia, with very low levels in the resting state and markedly greater activity in response to physiological stimulation and various pathological insults. Seizure activity (status epilepticus) is especially effective in driving high levels of MMP-9 in the bran. Status epilepticus may also provoke development of epilepsy (epileptogenesis) and thus it is not a surprise that missing MMP-9 in KO mice impairs epileptogenesis, whereas excessive MMP-9 levels in transgenic rats and mice facilitate it. Recently, we and others have shown that non-specific MMP-9 inhibitors impair epileptogenesis and thus might be considered as potential drugs for this condition that at present remains uncurable.

Cathepsin activity in disease management

G. Blum[I]

[I]*The Hebrew University, Jerusalem, Israel*

Identifying the activity of specific molecular entities in diseases by molecular imaging may show the localization of pathologies, shed light on disease progression and suggest suitable treatments. The cysteine cathepsins proteases, play key roles in several types of human diseases as they are highly active in tumor-associated macrophages. Thus, cathepsin proteases were found to be valuable biomarkers and therapeutic targets for several inflammatory diseases and may serve as targets for molecular imaging. Over the last decade the Blum lab has been focusing on generating various types of selective activity-based probes (ABPs) targeted to cathepsin B, L and S, as tools to detect the activity of these enzymes in cells and in vivo. ABPs are small molecules designed to modify enzyme targets in an activity-dependent manner, covalently attaching a tag to their active site enabling detection of enzyme activity. Unpublished data on gold nano-particulate probes for CT imaging and their applications in cancer-setting will be described in the lecture. In addition, the use of cathepsin probes and inhibitors to detect and abrogate resistance to immunotherapy treatment will be presented. In conclusion, The ABP technology is unique, it enables studying the involvement of enzyme activity in diseases and has potential for clinical use.

Highly selective furin inhibition as a therapeutic approach for chronic obstructive pulmonary disease.

L.E.J. Douglas[I], J.A. Reihill[I], T. Odrljin[II], S.L. Martin[I]

[I]*School of Pharmacy, Queen's University Belfast, Belfast, United Kingdom*
[II]*Boston Pharmaceuticals, Cambridge, MA, United States of America*

Chronic obstructive pulmonary disease (COPD), associated with two major clinical phenotypes; chronic bronchitis (CB) and emphysema, is the third leading cause of death worldwide. Symptoms of CB, to include excessive mucus production and impaired mucociliary clearance (MCC), are influenced by airways hydration which is regulated by Cl^- secretion by the cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+ absorption by the epithelial sodium channel (ENaC). Furin, a cellular proprotein convertase, is a key proteolytic activator of ENaC. We have recently shown highly selective furin inhibition to inhibit ENaC and improve MCC in cystic fibrosis (CF) airways cells (Douglas et al (2022) Cell Chem. Biol. 29(6):947-957). As mutated CFTR results in CF lung disease that shares phenotypic characteristics with CB, the aim of this study was to investigate the effect of a novel, highly potent, selective furin inhibitor, BOS-857 on ENaC activity and MCC in COPD. This study also aimed to determine whether BOS-857 used in combination with the CFTR potentiator, ivacaftor would provide additional benefit, as reduced CFTR activity is caused by cigarette smoke exposure, a common risk factor for COPD. BOS-857 treatment of COPD HBECs significantly reduced ENaC-mediated Na^+ absorption by 75% after a 48h exposure and prevented subsequent cell-surface activation of ENaC by neutrophil elastase. Treatment of COPD HBECs with either BOS-857 or VX-770 alone improved MCC rates. When COPD HBECs were treated with a combination of BOS-857 and VX-770, significant improvements in MCC rate over VX-770 alone were observed ($p< 0.05$). These studies support highly selective furin inhibition as a therapeutic approach for COPD through ENaC inhibition, which has the potential to drive airways rehydration and improve MCC. Further therapeutic benefit may also be derived by combining furin inhibition with a CFTR potentiator. This work was funded by a project grant awarded by Boston Pharmaceuticals.

Identification of a deubiquitinating enzyme (DUB) that regulates CFTR-modulator therapy in cystic fibrosis

J. Reihill[I], R. Wilkinson[II], I. Lobb[II], D. Longley[II], T. Harrison[II], S.L. Martin[I]

[I]*Queen's University Belfast, Belfast, United Kingdom*
[II]*Almac Discovery, Belfast, United Kingdom*

Cystic fibrosis (CF) is a life-limiting disease caused by loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a chloride ion channel. Loss of CFTR activity severely compromises mucociliary clearance (MCC), and this is the underlying pathogenesis of CF lung disease. New medicines that improve CFTR function include the triple combination therapy consisting of two CFTR correctors (Elexacaftor/Tezacaftor) that restore protein folding and a potentiator (Ivacaftor) that enhances channel opening. These therapies however, do not return channel activity to wildtype levels and are non-curative. Deubiquitinating enzymes (DUBs) play a central role in the trafficking and recycling of cellular proteins, yet have been under-investigated in CF. The objective of this study was therefore, to identify specific DUBs as potential therapeutic targets for CF drug development. An arrayed CRISPR-based genetic knockout screen targeting 94 individual DUBs was performed using a differentiated human primary bronchial epithelial cell (PBEC) model, followed by an electrophysiological assessment of ion channel activity. In non-CF PBECs, knockout of one specific DUB (here denoted as DUB-X) resulted in elevated activity of both CFTR and TMEM16A (a Ca2+-activated Cl- ion channel that can also promote MCC), with similar results obtained using a specific pharmacological inhibitor. In CF PBECs obtained from multiple donors both genetic knockout and pharmacological inhibition of DUB-X increased TMEM16A activity and augmented CFTR activity induced by the gold standard triple combination therapy. Importantly, the elevation in CFTR and TMEM16A-mediated Cl- secretion elicited by DUB-X inhibition corresponded with substantially improved MCC rates, which were assayed via a bead-tracking assay in differentiated primary PBECs. This study highlights DUB-X as an attractive target for next generation drug discovery and development in CF.

Cystatins in cancer progression: More than just cathepsin inhibitors

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[II]*Jozef Stefan Institute, Department of Biotechnology, Ljubljana, Slovenia*
[III]*UCLA, School of Dentistry, The Jane and Jerry Weintraub center for Reconstructive Biotechnology, Los Angeles, United States of America*

Cysteine cathepsins are involved in diverse processes of immune response, including the regulation of granule dependent cytotoxicity of cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells. The activity of cysteine cathepsins is regulated by cystatins which are in general cytosolic or extracellular proteins acting as emergency inhibitors, with exception of cystatin F which is present in endosomal/lysosomal vesicles and thus able to directly impair the activity of its targets. We tested whether cystatin F, derived either from immune or bystander cells in tumor microenvironment is able reduce NK and CTL cytotoxicity. Cystatin F is delivered to endosomal/lysosomal vesicles as an inactive, disulphide-linked dimer which is transformed to a monomer after proteolytic cleavage of 15 N-terminal amino acids. The truncated monomer is a potent inhibitor of cathepsins C, H and L, peptidases involved in the activation of granzymes and perforin from their precursors. The glycosylation pattern and the activation of M6PR pathway are important in controlling secretion of cystatin F from target cells, as well as internalization by cytotoxic cells and trafficking to endosomal/lysosomal vesicles. In tumor microenvironment, inactive dimeric cystatin F can be secreted from cancer stem cells, differentiated tumor cells and monocytes and is taken up by cytotoxic cells. Subsequent monomerization and inhibition of cysteine cathepsins within the endosomal/lysosomal vesicles impair granzyme and perforin activation, and induce cell anergy. Anergic NK cells, on the other hand, by increasing cytokine secretion direct cancer stem cells and monocytes towards differentiation. We propose cystatin F as a main mediator of the interplay between cancer stem cells, differentiated cancer cells and cytotoxic immune cells in tumor microenvironment. By decreasing its expression, modulating the glycosylation profile or by preventing monomerization we can improve anti-tumor immune response.

Discovery of small-molecule activators of NRF1 transcriptional activity preventing protein aggregation

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[II]*Institute of Organic Chemistry and Biochemistry, CAS, v.v.i., Prague, Czech Republic*
[III]*Palacky University, Prague, Czech Republic*

The intracellular accumulation of abnormal proteins causes proteotoxic stress that leads to pathological stages. When the accumulation of redundant proteins exceeds their degradation, undesirable signaling and/or aggregation occur, which are hallmarks of neurodegenerative diseases. This phenomenon occurs in parallel with the decline in proteasome activity. Due to the complicated structure of the 26S proteasome, its biogenesis must be strictly regulated at the levels of transcription, translation, and molecular assembly. NRF1 (encoded by the NFE2L1 gene) is a transcription factor that upregulates the expression of all proteasome subunits in a concerted manner, especially during stress conditions. Under normal conditions, it is embedded in the membrane of the endoplasmic reticulum, retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. However, when cell proteostasis is impaired, NRF1 is cleaved by the DDI2 protease and as a processed transcription factor, it switches on the expression of proteasome genes and other rescue factors. Therefore, activation of the NRF1 pathway could represent a new approach to delay the onset or ameliorate symptoms of neurodegenerative disorders and other disorders with disturbed proteostasis. Here, we present a series of small compounds that are able to induce NRF1-dependent proteasome synthesis and the heat shock response both in cell lines and in C. elegans model strains. Compounds increase proteasome activity and decrease the size and number of protein aggregates. Importantly, the compounds do not cause any cellular stress. Overall, our compounds represent a promising novel therapeutic approach for the treatment of a variety of protein conformational diseases, including the most debilitating neurodegenerative diseases.

Stefin B at the crosstalk of autophagy and inflammation

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Stefin B (cystatin B) is an inhibitor of nuclear and lysosomal cysteine cathepsins. A gene for stefin B is located on human chromosome 21 and is overexpressed in the brain of Down syndrome individuals. Loss of function mutations in the gene of stefin B are associated with the neurodegenerative disease known as Unverricht-Lundborg disease (EPM1), which is characterized by progressive myoclonus, epilepsy and ataxia. Stefin B-deficient mice were significantly more sensitive to the lethal LPS-induced sepsis and had enchanced NLRP3 inflammasome activation. In the present work we showed lower caspase-11 gene expression and lower IL-1β processing in bone marrow-derived macrophages from stefin B trisomic mice. We further showed that stefin B increased AMP-activated kinase activation and suppressed mTOR activity, accordingly it prevented mitochondrial ROS formation and impaired NLRP3 inflammasome activation. Collectively, our study demonstrates that the increased expression of stefin B induced autophagy and prevented inflammatory response. Our findings reveal the basis of anti-inflammatory properties of stefin B and may help to develop novel therapeutic approach to prevent excessive NLRP3 inflammasome activation.

A new family of cysteine and aspartic peptidase inhibitors with beta-trefoil fold from fungi

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[II]*Department of Biochemistry and Molecular and Structural Biology, Jozef Stefan Institute, Ljubljana, Slovenia*
[III]*Department of Biology, Institute of Microbiology, ETH Zürich, Zurich, Switzerland*

Peptidase inhibitors with β-trefoil fold (Clan IC in Merops) include the Kunitz type II inhibitors of plant origin (family I3) with representatives that inhibit serine, cysteine, and aspartic peptidases, as well as four families of inhibitors of fungal origin. These are mycocypins (families I48 and I85), which inhibit cysteine peptidases by a distinct inhibitory mechanism, mycospins (family I66), which include trypsin-specific inhibitors, and the newly described cocaprins (family I106), which contain double-headed peptidase inhibitors of cysteine and aspartic peptidases. Cocaprins (*Coprinopsis cinerea* cysteine and aspartic peptidase inhibitors) have a β-trefoil fold resembling a tree with typical features such as a six-stranded beta barrel stem and a crown formed by six β-strands and connecting loops. Cocaprins inhibit C1 family plant cysteine peptidases, papain and ficain, with K_i in the low micromolar range, but do not inhibit the C13 family peptidase legumain, which distinguishes them from mycocypins. In addition, unlike mycocypins, cocaprins do not inhibit the human cysteine peptidase, cathepsin L. Cocaprins also inhibit the aspartic peptidase pepsin of family A1, with K_i in the low micromolar range, but not the fungal aspartic peptidase rhizopuspepsin. Site-directed mutagenesis revealed that the β2-β3 loop is involved in the inhibition of cysteine peptidases and that the inhibitory reactive sites for aspartic and cysteine peptidases are located at different sites on the protein. The new fungal family of β-trefoil peptidase inhibitors further extends the incredible versatility of β-trefoil fold loop functionalization. Different inhibitory sites are recruited to inhibit the same peptidase, and the same inhibitory site can be recruited to inhibit different peptidases in different members of the IC clan. The plasticity of surface functionalization makes the β-trefoil fold an ideal scaffold for designer peptidase inhibitors.

Potpourri of cysteine cathepsins in health and disease of the thyroid gland

M. Rehders[I], A. Dogru[I], A. Al-Hashimi[I], V. Venugopalan[I], **K. Brix**[I]

[I]*Jacobs University Bremen, Bremen, Germany*

Cysteine cathepsins of thyroid epithelial cells are not restricted to endosomes and lysosomes. The cathepsins B, K, L and V become secreted upon TSH-stimulation for physiological thyroglobulin processing including thyroid hormone liberation at the apical pole of thyroid epithelial cells. In contrast, secretion of cathepsin B at the basolateral pole enables thyroid carcinoma cells to degrade extracellular matrix during tumorigenesis, while an N-terminally truncated form of cathepsin V is sorted to nuclei and promotes proliferation. Both, thyroid epithelial and carcinoma cells form primary cilia to which the cathepsins B, K and L localize. Treatment with cysteine peptidase inhibitors results in loss of cilia from the cells, and causes a redistribution of the thyroid co-regulating GPCR Taar1 from cilia to the endoplasmic reticulum. These data suggest that proteolytic activities of cysteine cathepsins at cilia are important to maintain sensory and signaling properties for regulation and homeostasis of thyroid follicles. Indeed, a lack of the cysteine cathepsin K and thyroid hormone transporters, essential to either liberate or release thyroxine from thyroid follicles, causes a vicious cycle of autophagy induction resulting in self-thyrotoxicity. We conclude that cysteine cathepsins of the thyroid gland are co-regulated with and by GPCRs and membrane transporter proteins. The enormous variety of locations in which cysteine cathepsins are active within and around thyroid epithelial cells allows them to co-determine vital tasks of the healthy thyroid gland. On the other hand, loss of cysteine cathepsins, active site mutations and/or mistrafficking contribute to onset and progression of thyroid diseases.

Exosome-mediated uptake of mast cell tryptase into the nucleus of melanoma cells: a novel axis for regulating tumor cell proliferation and gene expression

G. Pejler[I]

[I]*Uppsala University, Uppsala, Sweden*

It is well established that mast cell accumulation accompanies most malignancies. However, the knowledge of how mast cells functionally impact on tumors is still rudimentary. Here we addressed this issue and show that mast cells have anti-proliferative activity on melanoma cells and that this effect is dependent on tryptase, a tetrameric protease stored in mast cell granules. Mechanistically, tryptase was found to be endocytosed by melanoma cells as cargo of DNA-coated exosomes released from melanoma cells, followed by transport to the nucleus. In the nucleus, tryptase executed clipping of histone 3 and degradation of Lamin B1, accompanied by extensive nuclear remodeling. Moreover, tryptase degraded hnRNP A2/B1, a protein involved in mRNA stabilization and interaction with non-coding RNAs. This was followed by downregulated expression of the oncogene EGR1 and of multiple non-coding RNAs, including oncogenic species. Altogether, these findings establish a new principle for regulation of tumor cell proliferation.

Dissecting the role of Cathepsin V in breast cancer

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[I]*Queen's University Belfast, Belfast, United Kingdom*

Studies have shown that Cathepsin V exhibits elevated expression in breast cancer tissue and is associated with distant metastasis. Cathepsin V has previously been associated with ER positive breast cancers due to its inclusion on the Oncotype DX genomic test, where it is one of 16 cancer related genes used to assess the risk of ER positive breast cancer recurrence and to determine the benefits of adjuvant chemotherapy treatment. Research has also identified that Cathepsin V expression is elevated in tumour tissues from numerous other malignancies, but despite this there has been limited examination of the function of this protease in cancer. We have identified that elevated Cathepsin V expression is associated with reduced survival in ER positive breast cancers. Examination of MCF-7 and ZR-75-1 shRNA cell line models reveals that Cathepsin V facilitates proliferation and invasion of ER positive breast cancer cells. We have also determined that Cathepsin V regulates the protein expression of GATA3, through promoting its degradation via the proteasome. Cell signalling analysis has shown that depletion of Cathepsin V results in elevated pAkt-1 and reduced GSK-3β expression, which rescues GATA3 from proteasomal degradation. Collectively, our research has identified that Cathepsin V promotes ER positive breast cancer through facilitating tumour cell proliferation, invasion and suppressing the expression of GATA3. Cathepsin V has not previously been associated with the Akt-GSK-3β-GATA3 pathway and whether it facilitates the degradation of other proteins by the proteasome remains to be elucidated. These results suggest that Cathepsin V may represent a viable therapeutic target in ER positive breast cancer, however comprehensive analysis of in vivo models is necessary, particularly in relation to metastasis, which GATA3 has been shown to impair.

Colonic mucus proteolysis in colitis

E. Nyström[I], C. Becker-Pauly[I]

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Our intestinal surfaces are coated with a layer of mucus composed of networks of polymeric glycoproteins. This mucus fundamentally functions to protect our gut from environmental challenges, especially from infection by potentially pathogenic microorganisms. In the colon, the mucus acts as a barrier system that separates potentially hazardous factors in the intestinal lumen, primarily the microbiota, from the epithelial surface. A correlation between mucus barrier defects and intestinal inflammation, colitis, has been established in animal models, and emerging clinical evidence in humans shows that mucus barrier function is severely reduced in ulcerative colitis (UC), an emergent inflammatory bowel disease that specifically afflicts the colon. However, the mechanistic basis of the observed defects remains a mystery. Proteolysis is known to be critical for remodelling glycoprotein polymeric networks throughout the human body, and we have previously shown that the metalloprotease CLCA1 is important for regulating baseline mucus properties together with a yet unidentified cysteine protease. However, the involvement of mucus proteolysis in UC has not yet been specifically investigated. In dextran sodium sulphate (DSS) induced colitis, an experimental animal model of UC, mucus barrier defects were observed before onset of inflammation and coincided with altered protease activity in the mucus. These alterations included increased activity of Clca1 and induced secretion of the Ctss cysteine protease into the mucus. Further, both Clca1 and Muc2, the main glycoprotein in colonic mucus, were found to be Ctss substrates in vitro, indicating a potential role of Ctss as a key regulator of colonic mucus properties in the onset of inflammation.

Inflammatory caspases and crosstalk with apoptosis: Signaling mechanisms and role in disease

M. Lamkanfi[I]

[I]*Laboratory of Medical Immunology, Ghent, Belgium*

Inflammasomes are intracellular molecular platforms for activation of inflammatory caspases. They play an important role in the innate immune response to pathogens and their dysregulated activation causes a variety of autoinflammatory diseases. In addition to promoting maturation and secretion of interleukin(IL)-1ß and IL-18, inflammasomes induce an inflammatory cell death mode termed pyroptosis. Here, I will discuss recent insights revealing extensive crosstalk between inflammatory and apoptotic caspases, the signaling mechanisms involved and the role of inflammasome-induced cell death in inflammatory disease.

Cathepsin S and chronic obstructive pulmonary disease (COPD)

P. Bigot[I], S. Chesseron[I], A. Saidi[I], D. Sizaret[II], A. Petit-Courty[I], Y. Courty[I], F. Lecaille[I], **G. Lalmanach**[III]

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Smoking is accountable for more than 80% of chronic obstructive pulmonary disease (COPD). COPD is a progressive and irreversible heterogeneous disease, which encompasses emphysema, chronic bronchitis, small airway obstruction and/or fibrosis. COPD is associated with mucus hyper secretion and exacerbation episodes. Nearby the recruitment of cells that contribute to airway inflammation and aberrant lung tissue remodeling, cigarette smoke increases the permeability of lung epithelia during emphysema. Exposure of the lung to cigarette smoke elicits the upregulation of cathepsin S (CatS). Despite an oxidizing environment, the activity of CatS remains partially safeguarded. The unanticipated maintenance of the reactivity of the nucleophilic Cys25 within the active site depends on the reversible formation of a sulfenic acid, followed by a slower conversion to sulfinic acid. Here signaling pathways triggering increased expression of CatS by macrophages were examined. Likewise, we studied molecular mechanisms sustaining the potential modulation of the epithelial integrity by CatS. Our objective was to assess the impact of CatS on the permeability of lung epithelial cells and to identify CatS target(s) of clinical significance. Taking into account the deleterious elastinolytic activity of CatS favoring emphysema, our data strengthen the therapeutic relevance of targeting CatS in COPD.

Emerging role of legumain in innate immune response – potential link to anti-inflammatory effects

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Legumain is an evolutionary conserved lysosomal protease with a distinct cleavage selectively for an internal asparagine or aspartate residue of a peptide backbone via a catalytic cysteine residue. Legumain is most abundant in the kidney, placenta, spleen, liver, testis, and thymus. Mice lacking legumain accumulate a discrete set of proteins in the end-lysosomes of their kidney proximal tubular cells (PTCs), suggesting a role in the normal catabolism of proteins in PTCs. Broad evolutionary conservation suggests important roles of this unique enzyme in lysosomal function and organ homeostasis but lack the molecular explanation for the resulting phenotype. To address this question we aimed our research to discover legumain proteolytic substrates and subsequently to identify legumain signaling pathways. In our work we investigated legumain knock out mice by performing a global proteomic analysis of several mouse tissues/cell types. Identification and relative protein quantification revealed a small group of lysosomal proteins significantly upregulated in legumain knock out mice. We additionally implemented a two-dimensional peptide visualization approach that provides semi quantitative topographical maps of proteins in the examined samples. With this approach we were able to screen for potential substrates on a set of several thousand identified proteins. We determined a handful of physiological substrates, partially confirming known targets (cathepsin L, H) and importantly, identified a set of previously unknown substrates. Novel targets show a link between legumain, specific substrate cleavage events and native immune response providing further evidence for legumain as an important player in pathophysiological processes. In context with elucidating the link with innate response and anti-inflammatory effects of legumain, novel results on studies of Candida albicans infection of mice and studies on both mouse and human cell models will be presented.

Activity profiling of viral proteases from SARS-CoV-2

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In December 2019, the first cases of infection with a novel coronavirus, SARS-CoV-2, were diagnosed. Currently, there are very few effective antiviral treatments for COVID-19. To address this emerging problem, we focused on the SARS-CoV-2-Mpro and SARS-CoV-2-PLpro proteases that constitutes one of the most attractive antiviral drug targets. We have synthesized a targeted combinatorial libraries of fluorogenic substrates to investigate their substrate specificity. We used it to determine the substrate preferences of the SARS-CoV and SARS-CoV proteases. On the basis of these findings, we designed and synthesized potent inhibitors and for SARS-CoV-2-Mpro two activity-based probes, for one of which we determined the crystal structure of its complex with the SARS-CoV-2 Mpro. We visualized active SARS-CoV-2 Mpro in nasopharyngeal epithelial cells of patients suffering from COVID-19 infection. For SARS-CoV-2-PLpro we have determined crystal structures of two inhibitors that reveals their inhibitory mechanisms and provides a molecular basis for the observed substrate specificity profiles. We have also demonstrated that SARS-CoV-2 PLpro harbors deISGylating activity similar to SARSCoV-1 PLpro but its ability to hydrolyze K48-linked Ub chains is diminished, which our sequence and structure analysis provides a basis for. Together, this work has revealed the molecular rules governing Mpro and PLpro substrate specificity and provides a framework for development of inhibitors with potential therapeutic value or drug repurposing.

Impairment of E-cadherin functions by bacterial pathogens

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The intact epithelium represents the first effective barrier against bacterial pathogens. Investigating the mechanisms by which pathogens can compromise the protective barrier, a novel function for the bacterial serine protease and chaperone HtrA (high temperature requirement A) was identified. The Gram-negative class-I carcinogen *Helicobacter pylori* (*H. pylori*) serves as a paradigm for the functional analysis of HtrA since it is one of the most successful human pathogens infecting approximately 40% of the world's population. Extracellular *H. pylori* HtrA directly cleaves the human cell adhesion protein E-cadherin exposed on the surface of gastric epithelial cells allowing bacterial transmigration across the epithelial barrier. HtrA-mediated E-cadherin shedding is now considered to be an important step in pathogenesis. E-cadherin was the first identified HtrA substrate. Components of the tight junction complexes (occludin, claudin-8) and desmoglein-2 were recently discovered as novel HtrA substrates implying that HtrA targets all types of intercellular adhesion complexes to disrupt the epithelial barrier function and polarity. HtrA-mediated E-cadherin shedding has been also observed for a broad range of additional Gram-negative pathogens suggesting that it is a prevalent pathogenic mechanism. In Gram-positive bacteria, HtrA proteins strongly differ in their domain structure. Consistent with Gram-negative pathogens, *Listeria monocytogenesis* and *Staphylococcus aureus* efficiently cleave-off the ectodomain of E-cadherin during early phases of infection. E-cadherin shedding involves autoproteolytic processing of HtrA proteins as an activation step. In summary, E-cadherin cleavage in response to bacterial pathogens is an early step in pathogenesis; hence, pharmacological inhibition of HtrA represents an attractive strategy to combat bacterial infections.

The effect of protease inhibitors on the infections of human endothelial cells by SARS-CoV2 variants of concern (VoCs)

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Since its first identification in January 2020, SARS-CoV-2 underwent numerous mutations in its structural and non-structural proteins (NSPs) which appear to alter the virus into a less pathogenic version. Here, we investigated the effect of SARS-CoV2 variants on the fusion of human endothelial cells (ECs) and the expression of viral and cellular proteins. We also analysed the effect of various protease inhibitors on the viral infectivity and the expression of the pro-inflammatory marker, TNF-a. The formation of giant cells, which has been associated with its vascular pathologies, was reduced going from the WT and alpha-VoCs to the delta and omicron variants although the overall number of infected cells increased the delta and omicron VoCs. Viral components such as spike, nucleocapsid protein, and dsRNA were also reduced in the delta and omicron strains when compared to WT and alpha-SARS-CoV2. In addition, the expression of TNF-a, cathepsin K (CatK) was significantly reduced in the delta and omicron strains whereas ACE2 expression remained constant. This may indicate the reduced pathogenicity of the later VoCs despite increased infectivity. Cysteine protease inhibitors including the selective CatK inhibitor odanacatib (ODN) reduced viral-induced cell fusion, viral cell infection, and the expression of proinflammatory TNF-a. The equal potency of ODN and the general cysteine protease inhibitor E64d indicates that CatK is the predominant cathepsin in viral cellular uptake in ECs. Nirmatrelvir, the viral cysteine protease 3CLMpro inhibitor showed comparable inhibitory efficacy in WT, alpha, delta, and omicron infected ECs. However, the compound also revealed a significant inhibition of cathepsins, which may affect the adaptive immunity in patients.

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Schistosoma mansoni cathepsin C: from functional biochemical analysis to antiparasitic inhibitors

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Blood flukes of the genus *Schistosoma* cause schistosomiasis, a neglected parasitic disease that affects over 200 million people. Treatment relies on just one drug, and new therapies are needed. Our work is focused on the cysteine protease cathepsin C from *Schistosoma mansoni* (SmCC), which is involved in digestion of host hemoglobin, the most important source of nutrients. We demonstrated using functional proteomics that SmCC is present in blooddwelling developmental stages of *S. mansoni* infecting humans (eggs, schistosomula, and adults). Gut association of SmCC in adult parasites was shown by immunofluorescence microscopy. Further, we investigated regulation of SmCC activity by synthetic inhibitors. A library of peptidomimetics with a reactive tetrafluorophenoxymethyl ketone warhead was tested in a kinetic fluorescence assay against native and recombinant SmCC. The most potent inhibitors of SmCC activity were able to induce deleterious phenotypes in cultured schistosomes. Our results suggest that SmCC is a promising target for the treatment of schistosomiasis and SmCC inhibitors represent potential antischistosomal drugs.

Structure(s) of thyroglobulin: insight in hormone production

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Thyroglobulin is the protein precursor of thyroid hormones, which are essential for growth, development and control of metabolism in vertebrates. Hormone synthesis from thyroglobulin (TG) occurs in the thyroid gland via the iodination and coupling of pairs of tyrosines and is completed by TG proteolysis. The limitations of crystal growth of TG samples were surpassed by the progress in cryo-electron microscopy (cryo-EM), which enabled us to determine 3-dimensional atomic structure of human thyroglobulin at ~3.5 Å resolution. Combining literature data, insight in positioning of proximate tyrosine residues, expression and site directed mutagenesis of thyroglobulin in HEK cells we were able to identify and confirm all hormonogenic tyrosine pairs. Structure analysis revealed that proximity, flexibility, and solvent exposure of the tyrosines are the key characteristics of hormonogenic sites. The coupled, iodinated tyrosine residues were however not present in our human TG structures, however, a later work by Kim et al. (Acta Cryst D, 2021) visualized two pairs of well resolved coupled tyrosine residues in the ~2.6 Å cryo-EM structure of natively iodinated bovine TG.

DPP9 and repair of DNA double-strand breaks

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N-terminal sequences are important sites for posttranslational modifications that alter protein localization, activity, and stability. Dipeptidyl peptidase 9 (DPP9) is a serine aminopeptidase with the rare ability to cleave off N-terminal dipeptides with the imino acid proline in the second position (Xaa_1-Pro_2). Here, we identify the tumor-suppressor BRCA2 as a DPP9 substrate, and show that DNA damage induces the interaction between DPP9 and BRCA2. We present crystallographic structures documenting intracrystalline enzymatic activity of DPP9, with the N-terminal Met_1Pro_2 of a $BRCA2_{1-40}$ peptide captured in its active site. Intriguingly, DPP9-depleted cells are hypersensitive to genotoxic agents caused by mitomycin C, radiation, or olaparib. Using reporter assays, we show that DPP9-silenced cells are impaired in the repair of DNA doublestrand breaks by homologous recombination. Mechanistically, DPP9 targets BRCA2 for proteasomal degradation, limits BRCA2 interaction with PALB2, and promotes the formation of RAD51 foci, the downstream function of BRCA2. Nterminal truncation mutants of BRCA2 that mimic a DPP9 product phenocopy the reduced BRCA2 stability and rescue RAD51 foci formation in DPP9-deficient cells. Taken together, we present DPP9 as a regulator of BRCA2 stability and propose that by finetuning the cellular concentrations of BRCA2, DPP9 alters the BRCA2 interactome, providing a possible explanation for DPP9's role in cancer.

Engineering a membrane-independent human prothrombinase through parsimonious mutation of factor Xa

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Thrombin is generated from prothrombin through sequential cleavage at two sites by the enzyme complex prothrombinase, composed of a serine protease, factor (f) Xa, and a cofactor, fVa, on phospholipid membranes. The strict order of cleavage is first at Arg320 to produce the active intermediate meizothrombin, followed by cleavage at Arg271 to release thrombin. Since thrombin is the effector enzyme in blood coagulation, a structural understanding of the assembly and function of prothrombinase is of critical importance. The affinity of fXa to fVa is low, with assembly dependent on a membrane surface, making structural studies difficult. However, prothrombinase from the venom of type C or D snakes do not require membranes. Type C delivers a high affinity complex of fVa and fXa, and type D delivers a fXa that binds to host fVa with high affinity. Our group previously reported the crystal structure of the type C venom prothrombinase from P. textilis. With the goal of designing a membrane-independent human prothrombinase, we used the structure to model the complex between a type D fXa (HopD) and human fVa. HopD binds to human fVa with nM affinity and cleaves prothrombin through the meizothrombin intermediate. Active site blocked HopD inhibits human prothrombinase with a Ki similar to the Kd, suggesting a similar binding site of HopD and human fXa on human fVa. Based on the predicted interface between fXa and fVa, we made chimeras of human fXa with three catalytic domain loops swapped out for the sequences of HopD. The individual loop swaps had little effect on affinity, but the combined chimera bound about 2-fold more tightly with a Kd of 740nM. Surprisingly, the additional swap of the EGF2 domain for that of HopD reduced the Kd to 47nM, and resulted in exclusive processing down the meizothrombin route. Analysis of the interface suggests only a handful of mutations in human fXa can confer high affinity for fVa.

Combining proteomic data and structural analysis reveals interplay of cysteine cathepsin structure rigidity and flexibility on selectivity of substrates

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Cysteine cathepsins are endosomal proteases that are predominantly involved in protein turnover with various specific roles in physiological and pathological processes. Their shared roles and broad specificities make it difficult to understand their functional differences. To address this problem proteomic analysis of cell lysates incubated with human cathepsins K, V, B, L, S and F was performed. It provided 30 000 protein cleavages, which analysis revealed heterogeneous substrate positions with non-normal distribution of amino acid residues and homogeneous positions with normal distribution. The heterogeneous positions were used to generate clusters which enabled the selection of training datasets for the generation of support vector machine models that were applied successfully in prediction of cleavage sites of the SARS-CoV-2 S protein. To get insight into the structural basis of cathepsin specificity, 30 peptidyl sequences of cathepsin V substrates were chosen. 20 crystal structures of their complexes with cathepsin V were determined at resolutions from 1.5 to 2.1 Å. Structural analysis showed that the heterogeneous positions are structurally restrained, whereas residues at homogeneous positions exploits structural variability of the protease. Comparison with variability observed in the crystal structures of cathepsins K and S is consistent with this interpretation. Biochemical analysis of proteolytic degradation of peptides performed by cathepsins L, K, and V in solution exposed multiple cleavage sites and also several differences between peptide and protein cleavages. Taken together, specificity and promiscuity of substrate binding is explained by the restraining substrate binding interactions resembling the lock and key mechanism, which are complemented by the induced fit and conformational variability of the rest of the binding region. This hint could be used in drug discovery.

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Potent inhibition of matriptase by serpin A5 is abrogated by heparin-binding

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Serpin A5 (SA5), also known as protein C inhibitor and plasminogen activator inhibitor-3 (PAI-3), is a serine protease inhibitor principally reported as a heparin-dependent inhibitor of proteases involved in coagulation and fibrinolysis, to include thrombin and factor Xa. High concentrations of SA5 (3-4 mM) in human seminal plasma and inhibition of kallikrein-like protease, prostate specific antigen (PSA) and acrosin indicate a further role in the regulation of fertilisation. Given its broad specificity and wide tissue distribution, the aim of this study was to investigate the ability of SA5 to inhibit other physiologically relevant trypsin-like proteases, such as the type II transmembrane serine proteases (TTSPs). Recombinant human TTSPs (human airway trypsin-like protease (HAT), hepsin and matriptase) as well as prostasin, a glycophosphatidylinositol-anchored membrane protease, were screened in the absence and presence of heparin using peptide-based fluorogenic substrate activity assays. Novel interactions were further probed using SDS-PAGE and western blot analysis, supported by molecular modelling studies. SA5 was found to be an effective inhibitor of HAT, hepsin and prostasin however, in contrast to thrombin for which the pIC50 increased from 5.9 to 7.2, no further enhancement of inhibitory activity against these proteases was observed in the presence of heparin. Of note, potent inhibition of matriptase (pIC50 7.5) by SA5 was observed (complex formation confirmed by SDS-PAGE and Western blotting), which was abolished when SA5 was first bound by heparin. Several structural differences between thrombin and matriptase related to heparin binding and identified by molecular modelling help explain these results. This is the first report of SA5 as a potent inhibitor of matriptase. It is possible that physiological regulation by heparin serves as a molecular switch that shifts the inhibitory activity of the protein from one protease target to another.

Protease signaling in contact inhibition of proliferation (CIP)

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Historically identified as simple protein degraders, proteases are now regarded as critical signaling scissors that modulate protein activity and control cell homeostasis, survival and death. Due to implications in numerous important biological processes, altered proteolysis occurs in most pathologies, including cardiovascular and neurodegenerative disorders, chronic inflammation and cancer. Using discovery and targeted degradomics, we have recently unraveled a novel intracellular function of HtrA serine peptidase 1 (HtrA1) in cell density-dependent survival signaling in human dermal fibroblasts. This indicated a decisive system-level function of limited proteolysis in cell density control and contact inhibition of proliferation (CIP), a pivotal mechanism to inhibit uncontrolled cell proliferation and cancer progression. Therefore, we used TMT-based terminal amine isotopic labelling of substrates (TAILS) to record proteolytic signatures of human dermal fibroblast undergoing CIP. By analyzing proteomes from cells at different cell confluences, we mapped time-resolved protein abundances and correlated these cellular adaptations with differential proteolytic events during the transition from proliferation to cell cycle arrest. In addition to charting out the proteolytic landscape in CIP, we identified calpain 1 as a major protease that together with HtrA1 modulates signaling pathways at increased cell densities by cleaving high mobility group protein HMG-I/HMG-Y (HMGA1), binding immunoglobulin protein (BiP) and zyxin. These new calpain 1 substrates all have been associated with regulation of cell survival and proliferation, indicating a novel role of calpain 1 in cell density control.

Interference of proteases with the oncogenic PI3K-pathway in breast cancer cells

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An oncogenic activation of the Phosphoinositde-3-kinase (PI3K)-signaling is the most common molecular event in human breast cancers. Yet, proteases display important but very diverse roles in this disease. Therefore we set out to investigate functional and molecular connections between proteases and the PI3K-AKT-mTOR signaling axis in breast cancer. A hypothesis-driven approach to tackle this task was based on the fact that the active mTORC1 complex needs to correctly assemble at the cytosolic surface of the lysosomal membrane. Therefore we tested the effect of lysosomal protease deficiencies, i.e. cathepsin D and cathepsin L knockout, on mTORC1 complex assembly, signaling and its effect on tumor growth. In fact, cathepsin deficiency led to lysosomal storage, mTORC1 disassembly and, therefore, reduced mTOR signaling outputs. In consequence cathepsin deficient mammary epithelial cells were strongly delayed in their oncogenic transformation and tumor formation in the transgenic MMTV-PyMT breast cancer mouse model. In a second approach to address the protease-PI3K-pathway interactions, we screened by unbiased shRNA-based genetic targeting of proteases in combination with clinically tested PI3K inhibitors. We identified 181 protease transcripts that influenced susceptibility of murine breast cancer cells to low dose PI3K inhibition. Employing independently generated inducible knockdown cell lines we validated 12 protease hits in breast cancer cells. In line with the known tumor promoting function of these proteases we demonstrated the deubiquitinating enzyme Usp7 and methionine aminopeptidase 2 (Metap2) to be important for murine and human breast cancer cell growth and discovered a role for methionine aminopeptidase 1 (Metap1) in this context. Most importantly, we demonstrated that Usp7, Metap1 or Metap2 knockdown combined with simultaneous PI3K inhibition resulted in synthetic lethality of murine and human breast cancer cells.

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The role of rhomboid protease RHBDL2 in epidermal growth factor receptor (EGFR) regulation in human keratinocytes

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RHBDL2 is a serine intramembrane protease of the rhomboid superfamily. Rhomboids are conserved across the tree of life and influence many important cellular processes such as signaling and membrane protein quality control. Rhomboids proteolytically secrete EGFR ligands in *Drosophila*, while their role in mammalian physiology and pathophysiology remains elusive. Since *rhbdl2* gene is highly expressed in human skin, lung and digestive tract, we have focused on human keratinocytes as a model for a major skin cell type. We have analysed the substrate repertoire of endogenous human RHBDL2 in spontaneously immortalized aneuploid keratinocytes (HaCaT cells) by quantitative proteomics of the secretome, and identified the receptor-tyrosine kinase EGFR as a major endogenous substrate. EGFR activation by soluble extracellular protein ligands profoundly impacts cell proliferation and differentiation. RHBDL2 activity sheds the EGFR ectodomain from the cell surface, which then acts as a soluble secreted decoy receptor. Its extracellular accumulation titrates down the available pool of extracellular ligands of the EGFR, thus dampening the basal activity of cellular EGFR. In addition, shedding of EGFR ectodomain by RHBDL2 is strongly activated by elevated calcium concentration, a condition typical for differentiating keratinocytes. Importantly, both effects occur in hTERT immortalized human keratinocytes and primary adult normal human epithelial keratinocytes, implying that RHBDL2 is an endogenous regulator of EGFR activity in keratinocytes. We have developed ketoamide inhibitors of RHBDL2, which can be used to suppress RHBDL2 activity in primary cells where genetic silencing may be difficult. Our findings indicate that RHBDL2 may influence keratinocyte differentiation, and consequently may have impact on skin barrier homeostasis.

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Assessment of protease activity using recombinant Probody Therapeutics

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Proteases are involved in the control of numerous physiological processes, and their dysregulation has been identified in a wide range of pathologies, including cancer. Protease activity is normally tightly regulated post-translationally and therefore cannot be accurately estimated based on mRNA or protein expression alone. Utilizing the unique features of our protease-activated antibody prodrug technology, Probody® therapeutics (Pb-Tx), we have developed a set of methods designed to assess protease activity in situ and in vivo. A zymography-based technique, the IHZ(TM) assay, enables visualization of protease activity through the protease mediated binding of Pb-Tx to a tissue target. This in situ zymography method was evaluated in xenograft tumor samples using protease specific substrates and inhibitors, and the correlation of the assay readout with the in vivo efficacy was demonstrated. Additionally, a novel quantitative ex vivo zymography (QZ) technology was developed to measure protease activity in biological tissues utilizing a capillary electrophoresis approach for the detection of activated Pb-Tx in a target-agnostic manner in pre-clinical and clinical tissue samples. We also developed imaging techniques using the unique capability of a Pb-Tx to bind its target after proteolytic cleavage to image protease activity in vivo utilizing near-infrared (NIR) fluorescent imaging and positron emission tomography (PET) modalities. With the ability to tailor Pb-Tx substrate specificity for diverse proteases these developed approaches offer the potential to increase our understanding of protease activity in tissues and inform diagnostic and therapeutic development for diseases, such as cancer, that are characterized by dysregulated proteolysis.

Remarkable potential of unnatural amino acids for the development of protease-responsive prodrugs

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Over last decade, antibody-drug conjugates have gained considerable academic and clinical attention due to their high therapeutic efficacy and improved safety profile compared to standard chemotherapy. To date all marketed protease-responsive ADCs contain Val-Cit or Gly-Gly-Phe-Gly sequences that are allegedly targeted by cathepsin B. However, a growing body of evidence indicate that these sequences are non-selectively recognized by others proteases, leading to systemic toxicity. Therefore there is a great need for the development of new peptide linkers that will improve ADCs delivery and controlled release of the active payload at the tumor site. In this project, we seek to develop a ground-breaking chemical biology approach for the development of new generation of ultra-selective protease-activated ADCs. By leveraging a state-of-the-art HyCoSuL (Hybrid Combinatorial Substrate Library) technology we performed in-depth breast cancer activome screening in order to investigate the overall substrate specificity of cancer cells and to discover which cancer-associated proteases are the primary target for prodrugs development. HyCoSuL uses libraries of thousands of peptidyl fluorogenic substrates with natural and unnatural amino acids and it was originally designed for the manufacturing of potent and selective substrates, inhibitors and activity-based probes for proteases. In this work, we decided to unleash its remarkable potential and directly apply it for the development of protease selective linkers in anticancer prodrugs. Our results demonstrate that prodrugs bearing selective peptides display significantly higher activtiy and selectivity than reference prodrugs, which legitimizes the application of unnatural amino acids for further development of protease-activated prodrugs.

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New protein reactive centers against aspartic proteases

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Protein inhibitors of aspartic proteases are rare in nature, and only two of them have been structurally characterized so far in complex with a target protease. Here we present new crystal structures of cathepsin D in complex with three proteinaceous inhibitors: potato inhibitors pAPI-1 and pAPI-2 from the plant Kunitz family, and equistatin domain 2 (Eqd2) from sea anemone belonging to the thyropin (thyroglobulin type-1-like) family. The inhibitors show distinct designs of structural binding motifs that are based on disulfide-stabilized loops forming a network of interactions in the extended non-primed part of the enzyme active site. Our results will help in the development of biomimetic inhibitors of medicinally relevant aspartic proteases.

LIST OF POSTERS

The marine cyanobacterial metabolite gallinamide A and its analogues are potent inhibitors of SmCB1 drug target and effective anti-schistosomals

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Schistosomiasis, a parasitic disease caused by blood flukes of the genus Schistosoma, is a global health problem with over 240 million people infected. Treatment relies on just one drug, and new therapies are needed. Schistosoma mansoni cathepsin B1 (SmCB1) is a critical protease for digestion of host blood proteins and a validated drug target. Gallinamide A is a natural peptidic metabolite isolated from the extract of marine cyanobacteria. It interacts with cysteine proteases in a covalent irreversible manner as Michael acceptor. Here, we screened a library of more than 20 synthetic analogs of gallinamide A for inhibition of SmCB1 and identified inhibitors with low nanomolar potency. These compounds exhibited a strong suppression effect on live schistosomes in culture. Furthermore, we solved the high resolution crystal structure of SmCB1 in complex with gallinamide A and determined its binding mode. Our study provides a new structural template that can be exploited for the development of novel antischistosomal chemotherapeutics.

New approach for production of saturated fatty acids in cassava cell cultures as antibreast cancer agent

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Cassava plant is one of the major economical crops, involved in many industrial applications and therapeutic purposes such as suppression of cancer cell activity. The present work targeted to assess saturated fatty acids and their derivatives in cassava cell cultures. Materials and methods Stem explants of in vitro growing plantlets were induced for calli on MS-medium supplemented with 1 mg/l naphthaleneacetic acid+0.5 mg/l benzylaminopurine. Medium containing 5 mg/l 2,4 dichlorophenoxyacetic acid (2.4-D) and 0.2 mg/l benzylaminopurine was selected for callus productivity. The extraction adequacy of aprotic polar (ethylacetate) and nonpolar (chloroform, n-hexane) solvents was analyzed and evaluated by gas chromatography–mass spectrometry. Results and conclusion Chloroform callus extract contained mostly fatty acid methyl esters and fatty acid propyl esters. In contrast, n-hexane extract contained higher amounts of fatty acid constituents in free form, such as palmitic acid (23.55%). Ethylacetate extract included the highest value of lauric acid (28.34%) in free form as well other fatty acids such as caprylic acid (14.525%), capric acid (2.53%), and enanthic acid (6.41%). Ethylacetate extract conferred the optimal efficiency to suppress the breast cancer cell prevalence (2.63 μg IC50), followed by hexanoic extract (3.44 μg IC50), and then chloroformic extract (6 μg IC50) recording the least value for cancer cell propagation. Potential of cassava as one of the medicinal valuable plants should be promoted for health-boosting purposes.

Peptidyl α-ketoamide inhibitors of mitochondrial rhomboid protease PARL as chemical tools to modulate mitophagy in the context of Parkinson's disease research

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Rhomboids are serine intramembrane proteases that fulfill manifold physiological functions in all domains of life ranging from quorum sensing in bacteria to cell signaling and protein quality control in eukaryotes. The mitochondrial rhomboid protease PARL regulates mitophagy via the PINK1/Parkin pathway by cleaving PINK1 and PGAM5 in the inner mitochondrial membrane. As mutations in this pathway have been associated with Parkinson's disease, PARL is a potential drug target for modulating PINK1/Parkin-dependent mitophagy. Developed in our laboratory, the most potent and selective inhibitors of rhomboid protease available to date are peptidyl α-ketoamides modified by a hydrophobic substituent at the ketoamide nitrogen. By altering the peptide sequence and the substituent at the ketoamide nitrogen, they can be adapted to different rhomboid proteases. We employed a novel in vitro assay with cell-free translated rhomboid to develop ketoamide inhibitors targeting PARL. The generated ketoamide shows high potency against PARL in cellular assays and induces effects comparable to a PARL knockdown on the PINK1/Parkin pathway. Hence, peptidyl α-ketoamides provide a feasible chemical tool for the modulation of PINK1/Parkin-dependent mitophagy in the context of cell biological studies and the search for therapeutic strategies for Parkinson's disease.

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Combinatorial targeting of the PI3K oncogenic signaling pathway and methionine aminopeptidases in breast cancer

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A previous genetic screen in the lab focused on identifying proteases that act as synthetic lethal partners with simultaneous phosphoinositide-3-kinase (PI3K)/ mammalian target of rapamycin (mTOR) inhibition for targeted breast cancer therapy. Knockdown of methionine aminopeptidases (Metap) 1 and 2 by shRNAs in combination with PI3K pathway inhibition caused a significant reduction in growth of breast cancer cells compared to PI3K inhibition only. This observation led us to hypothesize that simultaneous pharmacological inhibition of PI3K and Metap1 and 2 might have a combinatory effect on the growth reduction of human and murine breast cancer cells. Thus, the long-term goals of this project are to discover the most effective drug concentrations for combinatory treatment and to define the underlying mechanism responsible for this phenomenon. Therefore, the human breast cancer cells MDA MB 231 (Triple-negative BC) and MCF 7 (Luminal A BC with PI3KCA mutation) as well as the murine breast cancer PyB6 cells (with activated PI3K/mTOR signaling) derived from MMTV-PYMT mice were chosen. For Metap inhibition, TNP 470 a natural inhibitor targeting Metap 2 was used in combination with widely used PI3K inhibitors BKM 120 and Alpelisib. The combination treatment with different concentrations of TNP 470 and BKM/Alpelisib in viability assays showed the best effect at a concentration range from 100nM to 10,000nM. Furthermore, colony formation assays showed reduced growth of all the three cell lines of combinatory versus single treatments. Cells without oncogenic PI3K mutation were, as expected, less sensitive to PI3K inhibition but, interestingly, more sensitive to TNP470. Finally, we will discuss possible mechanistic links of the Metap enzymes with the PI3K pathway and how combinations with protease inhibitors might improve the therapeutic index of established targeted cancer therapies.

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Cystatin C-deficiency is associated with increased susceptibility to LPS-induced sepsis and impaired autophagy in mice

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Cystatin C is a potent cysteine protease inhibitor that plays an important role in various biological processes including cancer, cardiovascular diseases and neurodegenerative diseases. It is ubiquitously expressed and secreted from various cell types and is abundant in body fluids. Cystatin C was shown to have a regulatory role in the expression and secretion of inflammatory mediators, such as nitric oxide, from immune cells upon stimulation with lipopolysaccharide (LPS) or interferon (IFN)-γ. Inflammasomes are multi-molecular platforms that recruit and activate inflammatory caspases-1 and -11, leading to the maturation and secretion of pro inflammatory cytokines interleukins (IL)-1β and IL-18 and pyroptotic cell death, thus contributing to innate inflammatory responses. In the present study we demonstrated that cystatin C-deficient mice were significantly more sensitive to the lethal LPS-induced sepsis. We examined the role of cystatin C-deficiency in Nlrp3 inflammasome activation and release of pro-inflammatory cytokines in BMDMs upon LPS and ATP stimulation. Our results show that cystatin C-deficient BMDMs secrete higher amounts of pro-inflammatory cytokine IL-1β due to increased caspase-1 and -11 activation upon Nlrp3 inflammasome activation that is not mediated by elevated activity of cysteine cathepsins. Cystatin C deficiency had no effect on NF-κB and MAPK signalling pathways, lysosomal membrane integrity, reactive oxygen species production and mitochondrial damage. We have shown that cystatin C-deficient macrophages present dysfunctional autophagy with a reduced level of autophagosomes and decreased autophagy induction upon LPS stimulation. Autophagy has an important modulatory role in inflammation. Our data suggest that the decreased autophagy in the absence of cystatin C leads to excessive inflammatory response, resulting in the increased sensitivity of cystatin C-deficient mice to the LPS-induced sepsis.

The role of S100A12 and TLR4 in assessment of disease activity in familial mediterranean fever (FMF) and juvenile idiopathic arthritis (JIA)

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Our aim was to investigate the possible relationship between the serum S100A12 and TLR4 levels, and the activity of Familial Mediterranean Fever (FMF) and Juvenile Idiopathic Arthritis (JIA) in accordance with the routine biochemical parameters. Furthermore, the effectiveness of these two biomarkers in distinguishing FMF from JIA. 69 children with FMF, 68 children with JIA, and 35 healthy children were included in this study. The patient groups were further divided into two subgroups according to their disease activities. S100A12 and TLR4 levels were measured by the sandwich ELISA. In the FMF patient group, serum S100A12 level was found to be statistically significantly higher than both in the JIA patient group and in the control group ($p=0.000$, $p=0.000$ respectively). In the JIA patient group, serum S100A12 level was found to be statistically significantly elevated than the control group ($p=0.000$), while it was found to be statistically significantly lower than in the FMF patient group ($p=0.000$). TLR4 levels were statistically significantly higher in the attack period compared to the attack free period in children with FMF ($p<0.05$). Although there was no relationship between S100A12 levels and disease activity, there is a clear correlation between the S100A12 and AIDAI (AutoInflammatoryDiseaseIndex) in attack free FMF patients ($r=0.612$ $p=0.000$). Children with active disease had higher S100A12 levels compared to those in remission. S100A12 was found to have a sensitivity of 77.9% and a specificity of 92.4% for the differentiation of FMF and JIA patients at a cut-off value of 136.86 ng/mL. Serum S100A12 and TLR4 levels were not found to be a potentially valuable biomarker for assessing disease activity. In accordance with these results of our study, we consider that S100A12 and TLR4 may be used as biomarkers in the diagnosis and follow-up of the disease in FMF and JIA, and also S100A12 and TLR4 play a crucial role in inflammatory processes seen in both FMF and JIA.

The involvement of cysteine cathepsins in complement system regulation in cancer

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Cancer cells have a modified cell surface and therefore can be recognized as foreign cells by the human immune system. This potentially results in elevated complement activation on the tumour cell surface, consequently leading to cancer cell destruction. However, cancer cells have developed mechanisms to evade elimination by the complement system. Many types of tumour cells increasingly express and secrete numerous proteases, e.g. cysteine cathepsins, into their tumour microenvironment. Only recently, extracellular cysteine cathepsins have been recognized as potent sheddases, enzymes that cleave extracellular domains of transmembrane proteins on the tumour surface. It is suggested that cysteine cathepsins target the complement binding partners. This ectodomain shedding may impede complement activation, resulting in a condition in which the complement system is insufficiently activated to fight tumour growth, thereby promoting tumour cell survival. Our preliminary experiments on several human tumour cell lines have shown that cysteine cathepsins, specifically human cathepsin L, V and S, reduce complement deposition due to the shedding or proteolytic removal of the complement-binding sites on the cancer cell surface. We are currently establishing a murine cell model to study and prove this in murine tumour cell lines using murine cysteine cathepsins. Next, we plan to use syngeneic breast cancer mouse models e.g. Polyoma Middle T (PyMT) and 4T1 to further evaluate the in vivo significance of cathepsins in complement activation. This research work will give a new insight into the molecular mechanism by which cysteine cathepsins blunt complement activation in cancer and thereby promote cancer cell survival, cancer progression, and tumour spread.

Cathepsin cleavage of EGFR affects intracellular signaling pathways

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Cysteine cathepsins can be secreted into the tumor extracellular microenvironment by several different cells, including cancer cells, endothelial cells, fibroblasts, and tumor-associated macrophages (TAM). Secreted cysteine cathepsins S and L were found to cleave ectodomains of several membrane-bound proteins in pancreatic cancer. Among the identified proteins, the epidermal growth factor receptor (EGFR) was identified as a cysteine cathepsin substrate candidate with high physiological relevance. EGFR belongs to a large group of receptor tyrosine kinases and it is involved in various cellular processes, such as cellular proliferation, migration, and cell death. This receptor is usually activated by ligand binding, such as epidermal growth factor (EGF). However, overexpression of EGFR or several receptor mutations, such as EGFRvIII, can lead to its autophosphorylation even without ligand binding. Proteolytic cleavage of the ectodomain of the receptor can also influence its activity, as shown for the serine protease matriptase-prostasin. In our work, we have confirmed cathepsin L mediated cleavage of EGFR and determined the possible cleavage site. Our results show that cathepsin L cleaves EGFR domain I, and a small part of domain II. This cleavage influences phosphorylation of these truncated forms of EGFR (t-EGFR) and causes constitutive activation, independent of EGF ligand presence. HeLa cells expressing t-EGFR show different cellular phosphorylation profiles and sensitivity to tyrosine kinase inhibitor Erlotinib. Moreover, t-EGFR remained insensitive to monoclonal antibody Cetuximab. Since EGFR is one of the critical anticancer drug targets, our findings could lead to a better understanding of EGFR signalling and possibly more effective strategies in anticancer therapy. Additionally, our results confirm extracellular cysteine cathepsins' increasingly recognized important role in cancer.

Cryo-EM structure determination of a complex between major histocompatibility complex class II molecules and invariant chain

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An important mechanism which alerts immune system to the presence of pathogens is known as endosomal pathway. It is conducted by a complex orchestration and cooperation between major histocompatibility complex class II (MHCII) molecules and endosomes/lysosomes in the professional antigen presenting cells. During this process a chaperone molecule known as invariant chain (Ii) is removed from the MHCII complex and pathogen antigenic peptides, generated by endosomal/lysosomal proteases, attach to the MHCII molecules and are presented at the cell surface. In endoplasmic reticulum (ER) MHCII α- and β-chains assemble into heterodimer and associate with Ii, forming presumably a nine-subunit complex consisting of three αβ dimers and li trimer. The stoichiometry of nonameric MHCII-Ii complex has been primarily reported, even so, pentameric and heptameric assembly of MHCII-Ii was also documented. From ER MHCII-li complex is transported into the late endosomal compartment, where it is further processed by the lysosomal cysteine proteases. Using cell biology studies the processing scheme of MHCII-Ii was ascertained quite well, still at the molecular level the detailed mechanism of its processing and its synchronization with antigen processing is not yet understood. Structural insight of MHCII-Ii complex will provide better understanding of MHCII maturation and loading, whereas biochemical insight in Ii processing may reveal importance of cysteine proteases. We set up a baculovirus expression system for production of the MHCII-Ii complex. In order to obtain a soluble protein complex we designed the constructs so that the transmembrane regions responsible for formation of the MHCII dimers and Ii trimers were replaced by the leucine zipper motifs that self-associate into corresponding oligomers. Using cryo-electron microscopy we have determined 3D structure of the MHCII-Ii complex, that reveals nonameric assembly and shows that Ii trimer is positioned in the center of the complex.

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Peptide Visualizer: a PROTOMAP-based tool for downstream analysis of MaxQuant data

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PROtein TOpography and Migration Analysis Platform (PROTOMAP) is a proteomic technique which integrates SDS-PAGE migration rates and LC-MS/MS data to reveal proteolytic processing in complex biological samples. In the original PROTOMAP protocol, a complicated configuration file must be written for each of these steps, which leaves a possibility for error. Additionally, the detection using ProLuCID (SEQUEST) is quite sluggish, and it is exceedingly challenging to incorporate post-translational modifications into the search. A free cross-platform easy-to-use Python 3 script was written to perform PROTOMAP analysis from results generated by MaxQuant. In contrast to the original PROTOMAP analysis where the user is able to interpret if a protein is proteolytically processed compared to control this script provides the user with additional quantitative information about peptides and allows for further interpretation. Visualization of protein features retrieved from the UniProt online database allows the user to predict which regions are and which are not expected to be detected e.g. the signal peptide. Quantitative information provides the user with an additional idea of whether a protein is up or down-regulated in a given condition.

A new cathepsin D targeting system based on liposome-bound pepstatin A

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Cathepsin D is an aspartic protease, overexpressed in many cancers, where it plays an important role in tumor development, progression and metastasis. While physiologically an intracellular protein, it is excreted to the extracellular matrix in pathological conditions, making it an appropriate target for drug delivery systems. Here we present the development and evaluation of a new delivery system for tumor targeting, based on liposomes, functionalized with pepstatin A – a natural peptide inhibitor of cathepsin D. We exploited its high affinity for cathepsin D and used it as a targeting moiety on the surface of liposomes. We then confirmed the ability of the newly developed system for in vitro inhibition of cathepsin D. In a further step, we applied the pepstatin A-conjugated liposomes to several cathepsin D expressing breast cancer cell lines and showed their binding to the cell surface, thus verifying their usefulness as a targeted drug delivery system.

CrataBL, a lectin from Crataeva tapia bark, inhibits proliferation, migration and invasion of SK-MEL-28 human melanoma cells

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Background: Melanoma, a skin cancer developed in melanocytes, is an important pathology due to its degree of metastasis and low chances of cure when diagnosed late. In tumors, proteolysis is exacerbated by tumor cell proteases and protease inhibitors found in legumes are potential candidates to control these events and prevent cancer progression. Aims: Investigate the effect of CrataBL and its peptides in cell-based assays of melanoma. Methods: CrataBL was extracted from the bark of the tree and purified by ion exchange and size exclusion chromatography. Cells (SK-MEL-28) were treated with CrataBL and its peptides in viability, proliferation, migration, invasion, and cell death assays. Results: CrataBL (100 and 200 μM) reduced more than 60% of the cell viability and demonstrated resistance to proteolysis in the proliferation of SK-MEL-28 as its effect is also observed after 72 h of treatment (inhibition greater than 90%). Also, 100 μM of the protein inhibited more than 80% of migration, and more than 100% of invasion and induced cells to enter in late apoptosis at 48 h. The peptide derived (Pep-27), although not effective in inhibiting viability, proliferation, and invasion prevented more than 80% of cell migration at 24 and 48 h and reduced the number of viable cells at 24 h. Pep-27 was also associated with vemurafenib (chemotherapy drug) and caused an increase in early apoptosis after 48 h. Pep-26, on the other hand, interfered with 60% of migration and induced early and late apoptosis within 24 hours of treatment. Conclusion: These results are promising to continue its test in other cell events related to hallmarks of cancer as adhesion and cell signaling (western blotting).

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Production and characterization of A Disintegrin and Metalloproteinase 10 (ADAM10): a protease involved in body homeostasis and cancer progression

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A Disintegrin and Metalloproteinase 10 (ADAM10) is a mammalian transmembrane protease involved in several functions, including body homeostasis, cancer progression, and Alzheimer's prevention [10.1038/s41388-018-0669-2]. Several studies have recently demonstrated its primary role in cancer invasiveness and chemoresistance due to its capacity to release adhesion proteins such as CD44, E-cadherin, N-cadherin, and L1 adhesion molecules, whose functions are correlated with tumoral cell migration and invasion [10.1038/s41388-018-0669-2]. Only limited data are, however, available on the biological role and death kinetic properties of ADAM10. This lack of knowledge mainly resides behind the difficulty to obtain a highly pure and functional recombinant ADAM10, since it is a transmembrane protein, coordinated with zinc and calcium ions and with 4 disulfide bridges [10.1016/j.cell.2017.11.014]. The production of the ADAM10 extracellular domain, the catalytically active domain, has been already described in a baculovirus system [10.1016/j.cell.2017.11.014]. The purpose of this research is to develop a method to express the extracellular domain of ADAM10 in a high amount and in a scalable way using E.coli. The protein is expressed as inclusion bodies which are solubilized using chaotropic and reducing agents. Several protocols have already been tested to refold the protein into its catalytically active form. The production of a high quantity of ADAM10 extracellular domain represents a step forward in the understanding of which molecules interfere with its homeostatic role or with its Alzheimer's prevention functions. Moreover, since it has been demonstrated a role in cancers, this new way to produce ADAM10 could be used to elucidate the mechanism behind chemoresistance and invasiveness. Furthermore, due to metalloproteases' high identity, this procedure could be used also to produce and purify other metalloproteases such as ADAM17.

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Immunomodulatory cathepsin B from the house dust mite Dermatophagoides farinae: functional and structural characterization

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Mites are a major source of allergens and contributor to the rising incidence of allergic diseases, including bronchial asthma, rhinitis, and atopic dermatitis. Digestive enzymes produced by mites and released into the environment are potent allergens and a target for the treatment of allergic hypersensitivity. We performed the first detailed profiling of digestive proteolytic enzymes in the house dust mite Dermatophagoides farinae using functional proteomics and identified cathepsin B (DfCB) as a new major component protease. Recombinant DfCB was produced in the yeast expression system and enzymologically characterized. Furthermore, the purified DfCB was crystallized, and its preliminary 3D structure was solved by X-ray crystallography.

Novel chemical strategy for the development of anti-cancer antibody-drug conjugates activated by serine proteases

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Cancer became one of the most common diseases and despite the significant research effort that has been made in past decades, it still causes near 8 million deaths worldwide each year. Common cancer treatments include chemotherapy, immunotherapy, surgeries, photodynamic therapy and the most important – the right selection of personalized treatment. Antibody-drug conjugates (ADCs) are one of the fastest growing therapeutic segments in oncology. This system enables the precise delivery of cytotoxic drug to the cancer cells and the release of a free drug upon intra-tumoral internalization. The classical ADC is comprised of an antibody that targets antigens highly expressed on tumour cells but not on non-malignant cells, a cytotoxic payload that efficiently kills the cells and cleavable or non-cleavable linker that is a bridge between the antibody and the drug. In this project, we propose a new approach for the development of protease-responsive linkers that could improve the overall ADCs therapeutic efficacy by levaraging their potency, selectivity and safety profile. By using HyCoSuL (Hybrid Combinatorial Substrate Library) developed by our group in 2014, we are able to develop optimal peptide linkers for serine proteases that are upregulated in cancer cells. In order to detect cancer-associated serine proteases we performed a broad profiling of the cancer activome, defined as a set of catalytically active enzymes, using fluorescent substrate libraries. In this work, we present our screening data using HyCoSuL library towards Bt-474, MDA-MB-231, MCF-7 cell lines as well as matriptase I. Based on these results we designed and synthesized first-in-class prodrugs containing unnatural amino acids that are selectively activated by cancer-associated serine proteases.

Cathepsin D mediated protein turnover in neuronal differentiation and neurodegeneration

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The neuronal ceroid lipofuscinosis type 10 (CNL10) is an inherited neurodegenerative pediatric disease, which is caused by a defective cathepsin D (CTSD) – a lysosomal protease widely abundant in many tissues. Although CNL10 genetic causes and the resulting phenotype were previously described in detail, little is known about the about protein turnover in CTSD-deficient differentiating and differentiated neurons. LUHMES cells were chosen as neuronal model system, because these immortalized human mesencephalic stem cells carry a TET-off v-myc vector and can be differentiated into mature dopaminergic neurons in as little as 5 days by inactivating the myc-oncogene by tetracycline and addition of some neuronal growth factors. CTSD was inactivated by the aspartic protease inhibitor Pepstatin A. Initial fluorescence-microscopy and flow-cytometry experiments using Lysotracker™ showed an accumulation of lysosomes in inhibitor-treated cells during maturation. To determine protein turnover, we used a SILAC-based pulse-chase approach, with a switch in from C^{13}Lys/Arg to $C^{13}N^{15}$Lys/Arg at the start of the chase. Protein turnover was investigated the first two days and from day 4 to day 6 of LUHMES differentiation; both in presence of pepstatin A or solvent. Mass spectrometry allowed to identify and study the turnover of 1345 and 1560 proteins during early and late stages of LUHMES differentiation, respectively. Interestingly overall protein degradation was slower in late differentiation as compared to the initial differentiation stage. Contra-intuitively, CTSD inhibited cells showed increased degradation of short lived proteins, which may be associated with the known increase of autophagy in CTSD deficiency. Nevertheless, there are also a number of proteins stabilized upon CTSD inhibition. Significantly (de-)stabilized proteins and affiliated cellular processes will be shown and their relevance for neuronal differentiation and neurodegeneration discussed.

Inhibition of cystatin F activation by cathepsin V inhibitor increases immune cell cytotoxicity

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Cysteine cathepsins C, H, and L are important mediators of granule-dependent cytotoxicity of natural killer cells and cytotoxic T lymphocytes as they enable activation of granzymes and perforin, which execute cytotoxic effects on cancer cells. Cystatin F plays a central role in the regulation of cathepsins in cytotoxic immune cells. This type II family cystatin can be translocated to endo/lysosomes or secreted and further internalized into bystander cells due to the glycosylation. In the lysosomes it is activated from inactive dimeric form to active monomer by cathepsin V, which cleaves 15 N-terminal amino acids from cystatin F. Cystatin F is normally expressed by immune cells, however, in tumor microenvironment it was found to be increased in also non-immune cells. The increased levels of cystatin F may contribute to the immunosuppressive status of tumor microenvironment. We evaluated the effects of cathepsin V inhibition on the cytotoxicity of immune effector cells NK-92 and TALL-104. After molecular docking and biochemical evaluation, we selected the most potent and selective ureido methylpiperidine carboxylate derivative as inhibitor of cathepsin V. Next, we tested the effect of the selected compound on cystatin F activation in cytotoxic immune cells. The cystatin F dimer-to-monomer ratio was increased after treatment with both broad-spectrum peptidase inhibitor E-64d and after treatment with the selective reversible cathepsin V inhibitor. As expected, treatment of immune effector cells with E-64d decreased cytotoxic function, as this inhibitor impairs the activities of cathepsins C, H, and L. However, treatment of cytotoxic cells with cathepsin V inhibitor increased their cytotoxicity. By targeting the activating protease of cystatin F cathepsin V, we can reduce the detrimental effects of cystatin F on cytotoxic cells in the tumor microenvironment as selective inhibition of cathepsin V prevents the monomerization and activation of cystatin F.

Phosphoproteomic analysis of legumain deficient mice

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Legumain, a member of cysteine proteinase family, is an asparaginyl specific protease. Typically, it is located in endolysosomal system, however, under specific physiological conditions, it can also be found in the cytoplasm, cell nucleus in the extracellular milieu. Legumain's amino acid sequence is highly conserved among many different species, pointing to its significance for the normal physiological function of organisms. Interestingly, legumain knock out mice show a very mild phenotype. They are viable and fertile with no behavioural abnormalities. Compared to their wild type counterparts, they have reduced body mass, irregular kidney function and hyperinflammation. However, since no system-wide studies of these animals have been carried out, the molecular basis for the observed phenotype is largely unknown. It has been shown, that levels of EGF receptor are significantly increased in legumain null mice. An effect that may cause global changes in cellular signalling. Apart from that, legumain could also be able to influence the function of other receptors and kinases. To reveal possible molecular interactions, explaining phenotype observed in legumain knock out mice, we employed phosphoproteomic to study changes in protein phosphorylation combined with immunological methods to validate selected target proteins while also measuring expression levels of certain proteins. Obtained results will enable us to explain legumain's role in the physiology of the organism and its possible involvement in the immune response.

Structure-function relationship of human cathepsin F

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Cathepsin F is a lysosomal cysteine protease with some distinct structural features compared to other human cysteine cathepsins. Two decades after its discovery, the enzyme still remains poorly characterized because of difficulties encountered in getting pure protein in sufficient amounts. Earlier studies showed that different fragments of human cathepsin F were expressed in various systems, i.e., *E. coli*, *P. pastoris*, mammalian cell lines and baculovirus expression systems, respectively. However, the enzyme was obtained either in a cleaved form, inactive and/or limited quantity not sufficient for further investigation. Therefore, a sequence-based bioinformatics approach was crucial to assess the suitability of the wild-type protein from cloning until 3D structure determination. Noteworthy, our systematic approach shows for the first time the bottlenecks that prevented earlier attempts to get this protein using different strategies and/or expression systems. Based on the above, we initially evaluated the standard approaches, namely, traditional cloning and ligation independent cloning and the subsequent expression in *E. coli*. However, even the most advanced options have not yet rendered success. On the other side, a cell-free protein expression system, enabled us to get the wild-type human cathepsin F for its further characterization. Based on the available crystal structure of the mature human cathepsin F, we evaluated the effect of the mutations found in patients with a lysosomal storage disorder, namely, the adult-onset neuronal ceroid lipofuscinosis, Type B Kufs disease (CLN13). The obtained results clearly show a destabilizing effect of all evaluated mutants (Gly51Arg, Gly182Ala, Ser208Leu), thus providing the structural basis for the detrimental effect observed in our functional studies.

Evaluation of variable new antigen receptors (VNARs) as a novel cathepsin S (CTSS) targeting strategy

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Amongst the lysosomal cysteine cathepsin family of proteases, cathepsin S (CTSS) holds particular interest due to distinctive properties including a normal restricted expression profile, inducible upregulation and activity across a broad pH range. Aberrant CTSS expression and activity has been demonstrated in a variety of pathologies, marking it out as both a biomarker and potential therapeutic target. To further elucidate CTSS biology and better understand its role in disease states, more selective inhibitors are required. Such inhibitors should not only possess exquisite target specificity but also retain functionality at lysosomal pH. One approach yet to be exploited is the application of Variable New Antigen Receptors (VNARs). VNARs are the smallest naturally occurring binding domains in vertebrates and possess distinct properties which make them attractive candidates as biologic inhibitors. Amongst these are their inherent stability and a structural predisposition to bind cryptic target epitopes, inaccessible to conventional biologics. Here, we describe the development and characterisation of anti-CTSS VNARs. VNARs were initially isolated from a VNAR phage display library screened against the CTSS proenzyme. Promising binders identified by phage ELISA were expressed periplasmically, with binding affinity confirmed by ELISA and SPR. Binding affinity was subsequently illustrated to be retained at lysosomal pH. Lead VNAR clones were shown to inhibit the activity of CTSS in fluorescence-based activity assays in a dose dependent manner. Further investigation indicated interestingly, that the VNARs could inhibit the activation of the CTSS proform to the mature enzyme. This was further demonstrated in in vitro cell-based assays utilising an intracellular VNAR expression system. This study exemplifies VNARs as novel CTSS binders, with potential utility as both tools for further investigating CTSS biology and as therapeutics.

Development of chemical sensor for non-invasive optical imaging of human neutrophil elastase

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Neutrophils are white blood cells, also known as polymorphonuclear (PMN) leukocytes. They are the most abundant cells of the immune system circulating in the human peripheral blood, being crucial effector cells in the innate immune response. They are the first line of defense against pathogens, using three antimicrobial mechanisms: phagocytosis, degranulation, and the release of nuclear material in the form of Neutrophil Extracellular Traps (NETs) in the process called NETosis [1].

The proteolytic enzymes, especially serine proteases, located in neutrophil's granules, play a key effector role in these cells. Neutrophil serine proteases (NSPs) contain human neutrophil elastase (HNE), proteinase 3 (PR3), cathepsin G (CG), and neutrophil serine proteinase 4 (NSP4). NSPs have a broad substrate specificity, which allowing for the hydrolysis of many endogenous substrates, including the cell surface receptors or chemokines. This makes NSPs the key signaling molecules that control many physiological processes such as apoptosis [2]. PMN plays numerous functions within the organism involving the serine proteases, and this leads to the hypothesis that among the entire population of neutrophils, there are subpopulations with different content of proteolytic enzymes.

To test this, we developed new, more efficient, and selective sensors for the determination of NSPs activity allowing non-invasive detection of NSPs. Our chemical sensors, fluorogenic substrates, that contain a pair of fluorophore and fluorescence quencher, characterize with high potency, selectivity, and cell membrane permeability, therefore they may be used for the further NSPs examination within the cells.

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Bone proteolysis to accommodate the tooth: Myb and metalloproteinases

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Alveolar bone is a critical component of the periodontal apparatus to anchor the tooth within the dentition and is essential for lifelong maintenance of the functional complex. Bone remodelling is accompanying the entire tooth germ growth towards eruption. Several molecular factors are known to participate in the process, new ones are still emerging. One of such candidates is Myb (c-Myb), recently reported in developing mandible and alveolar bone formation during osseointegration of dental implants. Myb is widely investigated in cancerogenesis and is critical for haematopoiesis. Due to the latter fact, Myb deficiency is prenatally lethal. Nevertheless, the onset of alveolar/mandibular bone is initiated prior to the survival limit (prenatal day 15). Our preliminary data indicated that Myb can impact the expression pathways of some metalloproteinases (Mmps). In order to follow this initial observation, the expression dynamics of Mmps and Myb gene was investigated during prenatal alveolar/mandibular bone development. Those being detected were further analysed in the wild-type and Myb deficient samples. Transcripts of Mmps-2, -9, -13, -14, -15 and -16 displayed mostly increasing expressions towards later prenatal stages while expression of Mmp-11 was decreasing. Myb expression massively increased at the latest investigated prenatal stage (day 18). Notably, the same dramatic elevation was observed in the case of Mmp-13 which was found downregulated in the Myb deficient bone. Since later k/o stages are not available this first indication about the interference of Myb with Mmp-13 expression pathway needs to be confirmed by additional in vitro experiments, recently in process. The knowledge about proteolytic factors in alveolar bone remodelling is of interest in basic research but also due to potential applications, particularly in bone healing and implantology.

The marine cyanobacterial metabolite gallinamide A and its analogues are potent inhibitors of SmCB1 drug target and effective anti-schistosomals

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Schistosomiasis, a parasitic disease caused by blood flukes of the genus Schistosoma, is a global health problem with over 240 million people infected. Treatment relies on just one drug, and new therapies are needed. Schistosoma mansoni cathepsin B1 (SmCB1) is a critical protease for digestion of host blood proteins and a validated drug target. Gallinamide A is a natural peptidic metabolite isolated from the extract of marine cyanobacteria. It interacts with cysteine proteases in a covalent irreversible manner as Michael acceptor. Here, we screened a library of more than 20 synthetic analogs of gallinamide A for inhibition of SmCB1 and identified inhibitors with low nanomolar potency. These compounds exhibited a strong suppression effect on live schistosomes in culture. Furthermore, we solved the high resolution crystal structure of SmCB1 in complex with gallinamide A and determined its binding mode. Our study provides a new structural template that can be exploited for the development of novel antischistosomal chemotherapeutics.

Discovery of small-molecule activators of NRF1 transcriptional activity preventing protein aggregation

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The intracellular accumulation of abnormal proteins causes proteotoxic stress that leads to pathological stages. When the accumulation of redundant proteins exceeds their degradation, undesirable signaling and/or aggregation occur, which are hallmarks of neurodegenerative diseases. This phenomenon occurs in parallel with the decline in proteasome activity. Due to the complicated structure of the 26S proteasome, its biogenesis must be strictly regulated at the levels of transcription, translation, and molecular assembly. NRF1 (encoded by the NFE2L1 gene) is a transcription factor that upregulates the expression of all proteasome subunits in a concerted manner, especially during stress conditions. Under normal conditions, it is embedded in the membrane of the endoplasmic reticulum, retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. However, when cell proteostasis is impaired, NRF1 is cleaved by the DDI2 protease and as a processed transcription factor, it switches on the expression of proteasome genes and other rescue factors. Therefore, activation of the NRF1 pathway could represent a new approach to delay the onset or ameliorate symptoms of neurodegenerative disorders and other disorders with disturbed proteostasis. Here, we present a series of small compounds that are able to induce NRF1-dependent proteasome synthesis and the heat shock response both in cell lines and in C. elegans model strains. Compounds increase proteasome activity and decrease the size and number of protein aggregates. Importantly, the compounds do not cause any cellular stress. Overall, our compounds represent a promising novel therapeutic approach for the treatment of a variety of protein conformational diseases, including the most debilitating neurodegenerative diseases.

Calpeptin: dual inhibitor of human cathepsins and SARS-CoV-2 main protease

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COVID-19 caused by SARS-CoV-2 remains a significant global public health burden with scarce antiviral treatments of limited efficacy. A great need for specific antiviral drugs targeting various stages of viral cell entry and replication has led to our recent drug repurposing study that identified Calpeptin as a covalent active site inhibitor of SARS-CoV-2 main protease (Mpro), an essential cysteine protease for viral replication and a key drug target. Since host cysteine cathepsins mediate viral Spike-protein (S-protein) cleavage/activation upon endosomal cell entry and Calpeptin also inhibits cathepsin L, dual targeting of Mpro and cathepsins seems an attractive approach for SARS-CoV-2 inhibition. Furthermore, inverse results of *in vitro* Mpro inhibition assay and viral replication inhibition assay in cell cultures suggest that the majority of the observed inhibitory effect of Calpeptin on viral replication in cell cultures might be due to the inhibition of cathepsin L rather than Mpro. We have determined the inhibition of various cathepsins (L, V, K and B) by Calpeptin and derivatives, GC-376, S-Calp. For all tested compounds, inhibition of cathepsins in the picomolar range was observed, which is significantly lower than that for the inhibition of Mpro. The lowest Ki values were observed for the S-Calp inhibition of CatK and CatL with values of 50 pM and 148 pM, respectively. For GC-376, only slightly higher values of 91pM and 224 pM were detected. The Ki values for CatV were similar to CatL, whereas CatB values were in nM range. These values imply that in the cellular context several cysteine cathepsins, all endopeptidases, are inhibited much more effectively than Mpro at the same inhibitor concentration. Furthermore, the crystal structures of S-Calp-CatL and Calpeptin-CatV complexes revealed that the Calpeptin binds to cathepsins at the non-prime sites from S3 – S1 in a substrate like manner and forms covalent bond with catalytic Cys residue at the cleavage site.

Identification of extracellular substrates of caspases-3 and -7 in cancer

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Apoptosis, as one of the mechanisms of programmed cell death, is crucial for maintaining homeostasis and consequently impairments of apoptosis tip the balance from physiological to pathological conditions. For example, insufficient clearance of apoptotic bodies leads to the progression from immunologically silent to an immunogenic form of programmed cell death called secondary necrosis. Morphologically, the process is similar to pyroptotic cell death with disrupted membrane integrity and release of intracellular proteins to the extracellular space. Accordingly, caspase-3-like DEVDase activity has already been detected in the extracellular space of apoptotic cells. Additionally, reports show that some chemotherapeutic drugs cause cancer cell death with secondary necrotic or pyroptotic features. Consequently, we checked whether released active caspases could, similarly to cathepsins, perform selective proteolysis of membrane proteins from cancer cells. To investigate the extracellular role of executioner caspases-3, -7 we treated breast cancer cells with recombinant human caspases and used a mass-spectrometry proteomic platform to identify the proteins released from the cell surface. Furthermore, we confirmed the cleavage of the most commonly detected protein neuropilin-1 with immunoblotting. Target analysis revealed that caspases can cleave both cell adhesion molecules (e.g. CD44) and cell transmembrane receptors (e.g. NRP-1), but the exact consequences of these cleavages remain unknown. Additionally, we confirmed the presence of DEVDase activity in extracellular space during progression from apoptosis to secondary necrosis. Using immunoblotting we showed the presence of both caspases in the supernatants collected from apoptotic cells. In the future, we will try to validate whether the extracellular concentration of released caspases is sufficient to release extracellular domains of membrane-bound proteins and how this process affects the properties of cancer cells.

Schistosoma mansoni cathepsin C: from functional biochemical analysis to antiparasitic inhibitors

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Blood flukes of the genus *Schistosoma* cause schistosomiasis, a neglected parasitic disease that affects over 200 million people. Treatment relies on just one drug, and new therapies are needed. Our work is focused on the cysteine protease cathepsin C from *Schistosoma mansoni* (SmCC), which is involved in digestion of host hemoglobin, the most important source of nutrients. We demonstrated using functional proteomics that SmCC is present in blooddwelling developmental stages of *S. mansoni* infecting humans (eggs, schistosomula, and adults). Gut association of SmCC in adult parasites was shown by immunofluorescence microscopy. Further, we investigated regulation of SmCC activity by synthetic inhibitors. A library of peptidomimetics with a reactive tetrafluorophenoxymethyl ketone warhead was tested in a kinetic fluorescence assay against native and recombinant SmCC. The most potent inhibitors of SmCC activity were able to induce deleterious phenotypes in cultured schistosomes. Our results suggest that SmCC is a promising target for the treatment of schistosomiasis and SmCC inhibitors represent potential antischistosomal drugs.

Exploring redox regulation of the protease DPP9 using a novel genetically encoded DPP9 activity sensor

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The dipeptidyl peptidase DPP9 is involved in various pathways ranging from immune cell differentiation to neonatal development in mice. We identified a novel role of DPP9 in regulating the fate of mitochondrial proteins. Specifically, we demonstrated that the localization and stability of the DPP9 substrate protein AK2 – an adenylate kinase of the mitochondrial IMS – is dependent on DPP9 activity. While in vitro assays to analyze DPP9 activity are readily available, continuously monitoring DPP9 activity in living cells remains challenging. We developed a DPP9 activity sensor for application in cellular systems, which allows investigating the role of DPP9 in rather slow processes like differentiation. This sensor is based on the DPP9 cleavage sequence of AK2 in combination with fluorescent proteins. Previous studies proposed different kinds of redox regulation of DPP9 by providing in vitro data on the inhibition of DPP9 by addition of N-ethyl-maleimide, hydrogen peroxide or iodoacetamide. However, detailed insights into the underlying regulatory mechanisms are missing. Employing our DPP9 activity sensor we were able to analyze how DPP9 is affected by changes in the redox state of the cell. Our preliminary data suggests that the glutathione homeostasis has a significant impact on DPP9 activity. Given the presence of 14 cysteine residues in the DPP9 protein sequence, these findings imply a post-translational oxidation and/or glutathionylation of thiol groups in DPP9. In future we will focus our research on the physiological implications of DPP9 redox regulation as well as the application of the DPP9 activity sensor in immune or cancer cell lines to assess changes in DPP9 activity during the immune response, differentiation and upon other extraordinary conditions.

Interaction of murine cathepsin B and DARPin and its prospects

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Cysteine cathepsins are lysosomal proteinases that influence many cellular processes. They express as preproenzymes, get processed into proenzymes and are activated in the acidic milieu of lysosomes through cleavage of the propeptide by other active cathepsins. In healthy tissue, they are important for homeostasis and are involved in processes, such as bone remodelling, antigen processing and its presentation, thyroglobulin processing and protein turnover in general, including extracellular and adhesion proteins. They are known to overexpress and to be involved in the progression, invasion and metastasis of solid tumours as well but it is arduous to ascribe exact individual roles to individual members owing to the heterogeneity of tumours. Cathepsin B (CtsB) has been most studied in the context of cancer which can be located at the surface of invadopodia of tumour cells (association with annexin A2) as a proenzyme and in membrane invaginations (caveolin-1) where it was linked with extracellular degradation. Designed ankyrin repeat proteins (DARPins) are genetically engineered antibody mimetic proteins based on natural ankyrin proteins used for binding and involved in numerous cell functions. DARPins can be used as diagnostic or therapeutic agents due to their high specificity and affinity for the selected target. DARPin 4m3 shows a high affinity and selectivity for murine CtsB. Until now, the structure of murine CtsB has remained unresolved due to a lack of success in crystallization efforts but DARPin 4m3 was successfully used for chaperone-assisted crystallization and the crystal structure of the complex has been successfully resolved. Due to similar biochemical and physiological properties between human and murine CtsB, the structure of the mouse CtsB can offer important insight for structure-based drug design, especially if we consider the role of mice in the development of drugs.

Human stefin B alternative functions apart from inhibiting cathepsins

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We have studied folding and aggregation mechanisms of human stefin B mostly as a model protein. Later, we have followed its fate in cells upon over-expression and/or mutation. The propensity to form amyloid fibrils and pore-like oligomers was observed both in vitro and in cells. From these results we have derived some suggestions, how this protein could function apart from inhibiting cathepsins, such as an amateur chaperone function binding other amyloidogenic proteins, among them amyloid-beta. What comes first: protein aggregation and pore formation upon cellular stress in the form of heat, acidic and redox states or regulating oxidative stress and autophagy. Relevance to progressive myoclonus epilepsy - EPM1 will be given. Also, review of the finding by some other groups will be revealed, who point to involvement of stefin B in the synapse, in transcription and in vesicular transport.

Attenuation of the NF-κB mediated proinflammatory effect of kiwifruit cysteine protease Act d 1

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Many inflammatory events are regulated by the NF-κB signalling pathway including the allergic response to food allergens. Kiwifruit allergen Act d 1 is a cysteine protease that is known to disrupt the integrity of the epithelial barrier and to increase intestinal permeability but only in its active form, as we previously showed. Since the enzymatic activity of this protease triggers the NF-κB mediated inflammation process in both epithelial cells HEK293 and THP1 macrophages, we aimed to explore the inhibitory potential of selected small molecules on this signalling pathway in order to decrease the pro-inflammatory effect of food allergens. Fluorescent microscopy and flow cytometry were employed to confirm the activation of NF-κB in HEK293 cells after transfection with reporter NF-κB-GFP plasmid. After Act d 1 treatment, gene expression of pro-inflammatory cytokines in HEK293 and THP-1 macrophages significantly increased and showed a typical cytokine profile of allergic sensitization. Cells that were treated with vanillyl alcohol or lauric acid previous to the Act d 1 exposure showed decreased expression of cytokines (IL-1b, IL-6, IL-25, IL-33, TNFα). The transcription factor was activated upon Act d 1 treatment in the epithelial cells and subsequently attenuated by the small molecules. However, treatment with E-64 inhibited Act d 1 showed lack of NF-κB activation in the treated cells, thus emphasising the role of its enzymatic activity in the inflammation process. Small NF-κB inhibitory molecules like vanillyl alcohol and lauric acid possibly show promising role in the future of regulating the inflammation process in many diseases, in prevention of unwanted side effects and increasing safety of vaccines.

Non-canonical reactive site against serine proteases evolved on plant Kunitz inhibitors

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Protease inhibitors from the Kunitz family I3 are 20-25 kDa proteins widely distributed in the plant kingdom. They share a conserved β-trefoil fold in which variable loops are involved in interactions with proteases. Kunitz inhibitors target serine proteases using the canonical (Laskowski) mechanism based on a single binding loop with a conserved structure. Here, we present a set of high-resolution crystal structures of two potato Kunitz inhibitors in complex with serine proteases trypsin and chymotrypsin. The formation of binary and ternary complexes was analyzed by analytical ultracentrifugation. We identified a new, non-canonical type of reactive site that binds serine proteases with both trypsin and chymotrypsin specificities. It is formed by two separate loops interacting with the S1 and S1′ pockets of the enzyme. Through this structural mechanism, the non-canonical reactive site is stabilized against proteolysis by the target proteases, providing a functional advantage over the canonical design. The evolution of multiple reactive sites against serine proteases in the Kunitz family is discussed.

The expansion of CD4+ human T-cell clones leads to the expression of the cysteine peptidase inhibitor cystatin F

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CD4+ T cells with cytotoxic activity (CD4 CTL) have been detected in various immune responses, that suggests their role in protective and/or pathogenic immune functions. These cells have the ability to kill their targets in an MHC class II-restricted fashion and can secrete granzyme B and perforin. Therefore, CD4 CTL utilize the same cytotoxic effector mechanisms also utilized by CD8+CTL and natural killer (NK) cells. In vitro, during long-term cultivation, human CD4+T cells also gain cytotoxic functions. We have analysed, CD4+human T-cell clones derived from activated peripheral blood lymphocytes of healthy donors for the expression of cytotoxic machinery components. Cystatin F is an inhibitor of cysteine cathepsins, that regulates the cytotoxic efficacy of CD8+CTL and NK cells by inhibiting the major pro-granzyme convertases cathepsins C and H as well as cathepsin L, which is implicated in perforin activation. In this study, we show that human CD4+T-cell clones, after long term cultivation express the cysteine cathepsins involved in the activation of granzymes and perforin. More importantly, we show that they express both the inactive, dimeric and active, monomeric form of cystatin F. Similar to CD8+CTL, cysteine cathepsins C and H are the major targets of cystatin F in CD4 CTL clones. Moreover, CD4 CTL clones expressed the active forms of perforin and granzymes. The content of cystatin F decreased with time in culture concomitantly with an increase in the activities of granzymes. Thus, our results propose that cystatin F plays a role in regulating CD4 CTL cytotoxicity. Furthermore, as cystatin F can be secreted and taken up by bystander cells, our results suggest that CD4 CTL regulate immune responses also through cystatin F secretion.

[Perišić Nanut, M., Pawelec, G., Kos, J. Human CD4+ T-cell clone expansion leads to the expression of the cysteine peptidase inhibitor cystatin F. International Journal of Molecular Science, 2021, vol. 22, no. 16, 8408-1-8408-15

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