DEPARTMENT OF BIOCHEMISTRY, MOLECULAR AND STRUCTURAL BIOLOGY B-1

The research activities of the members of the B1 department are largely focused on studies of the physiological role of proteases and their endogenous protein inhibitors in normal and pathological states. The studies involve the mechanisms of protease action and the structural and functional properties of proteases and their inhibitors, as well as some other enzymes. Molecular mechanisms of protease action and regulation are only partially understood; therefore, a lot of work, especially to find more physiological substrates and the signalling pathways they regulate, remains to be done.

Head:
Prof. Boris Turk

Protease research has undergone a major expansion in the past decade, largely due to the extremely rapid development of new technologies, such as quantitative proteomics and *in vivo* imaging, as well as the extensive use of *in vivo* models. These have led to the identification of physiological substrates and resulted in a paradigm shift from the concept of proteases as protein-degrading enzymes to proteases as key signalling molecules. Their catalytic activities are precisely regulated, the most important ways being zymogen activation and inhibition by their endogenous protein inhibitors. Any imbalance in the regulation of proteases can lead to pathologies, such as autoimmune, cardiovascular, neurologic and neurodegenerative disorders, as well as cancer. Thus, proteases represent an extremely important group of targets for therapeutic intervention.

In the continuation of our research on the physiological roles of proteases, we investigated the role of the high-temperature requirement A (HtrA) protease secreted by the group 1 carcinogen *Helicobacter pylori*. Since the intact gastric epithelium is the primary target for *H. pylori* in the stomach, we aimed to identify the HtrA substrates on the cell surface of gastric cells. In this study we treated human gastric epithelial cells with recombinant HtrA and used LC–MS/MS to identify the membrane proteins released from the cell surface. We identified several extracellular membrane targets including desmoglein-2 (Dsg2), since Dsg2 is a component of desmosomal junctions and highly expressed in epithelial cells and cardiomyocytes. We therefore propose that Dsg2 cleavage by HtrA contributes to the disintegration of the gastric epithelial barrier in response to *H. pylori* infections (Bernegger et al., 2021). This research is part of a collaboration with prof. Silja Wessler (UNI Salzburg).

In collaboration with prof. Peter Hamar (Semmelweis University, Hungary) we have investigated the molecular changes elicited by modulated electro-hyperthermia (mEHT) using multiplex methods in an aggressive, therapy-resistant triple-negative breast-cancer model. In mEHT, a focused electromagnetic field is generated within the tumour, inducing cell death by thermal and nonthermal effects. With next-generation sequencing, nanostring and mass spectrometry we identified that mEHT induced the upregulation of the stress-related Hsp70 and cleaved caspase-3 proteins, resulting in the effective inhibition of tumour growth and proliferation. In addition, several acute stress-response proteins, including protease inhibitors, coagulation and heat-shock factors, and complement family members were among the most upregulated treatment-related genes/proteins. We also confirmed that the heat-shock factor inhibitor KRIBB11 reduced mEHT-induced complement factor 4 mRNA increase, indicating that the inhibition of this stress response is likely to enhance the effectiveness of mEHT and other cancer treatments (Schvarcz et al., 2021).

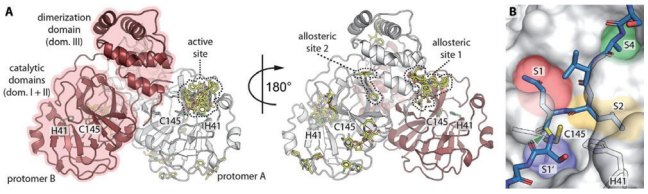

*Figure 1: X-ray screening of drug-repurposing libraries reveals compound binding sites distributed across the complete M^{pro} surface. (**A**) Schematic drawing of M^{pro} dimer structure. Protomer A in white, protomer B in red. For clarity, the 29 binding compounds (yellow sticks) are only depicted on one of the two protomers. Catalytic residues H41 and Cys145, active site and two allosteric drug binding sites are highlighted. (**B**) Close-up view of active site with peptide substrate bound (blue sticks), modelled after SARS-CoV M^{pro} (PDB 2Q6G). The scissile bond is indicated in yellow and with a green arrow. Substrate binding pockets S1', S1, S2 and S4 are indicated by colours.*

Our research on the endogenous inhibitors of proteases was primarily focused on inhibitors of cathepsins, stefins and cystatins. We thus explored the role of cystatin C, the major extracellular cathepsin inhibitor, in inflammation and sepsis (Biasizzo et al., 2021, Cells). We demonstrated that cystatin C-deficient mice (CstC KO) were significantly more sensitive to the lethal LPS-induced sepsis. We further showed increased caspase-11 gene expression and the enhanced processing of pro-inflammatory cytokines IL-1β and IL-18 in CstC KO bone-marrow-derived macrophages (BMDM) upon LPS and ATP stimulation. The pre-treatment of BMDMs with the cysteine cathepsin inhibitor E-64d did not reverse the effect of CstC deficiency on IL-1β processing and secretion, suggesting that the increased cysteine cathepsin activity determined in CstC KO BMDMs is not essential for NLRP3 inflammasome activation. The CstC deficiency had no effect on (mitochondrial) reactive oxygen species (ROS) generation, the MAPK signalling pathway or the secretion of anti-inflammatory cytokine IL-10. However, CstC-deficient BMDMs showed dysfunctional

autophagy, as autophagy induction via mTOR and AMPK signalling pathways was suppressed and the accumulation of SQSTM1/p62 indicated a reduced autophagic flux. Our study demonstrates that the excessive inflammatory response to the LPS-induced sepsis in CstC KO mice is dependent on increased caspase-11 expression and impaired autophagy.

Stefin B (cystatin B) is the major intracellular inhibitor of cathepsins, and the loss-of-function mutations in the stefin B gene were reported in patients with Unverricht–Lundborg disease (EPM1), a form of progressive myoclonus epilepsy. Stefin B-deficient mice, a mouse model of the disease, display key features of EPM1. Although the underlying mechanism is not yet completely clear, it was reported that the impaired redox homeostasis and inflammation in the brain contribute to the progression of the disease. In a study (Trstenjak et al., 2021, Antioxidants) we investigated whether lipopolysaccharide (LPS)-triggered neuroinflammation affected the protein levels of redox-sensitive proteins: thioredoxin (Trx1), thioredoxin reductase (TrxR), peroxiredoxins (Prxs) in brain and cerebella of stefin B-deficient mice. LPS challenge was found to result in a marked elevation of Trx1 and TrxR in the brain and cerebella of stefin B deficient mice, while Prx1 was upregulated only in cerebella after LPS challenge. Mitochondrial peroxiredoxin 3 (Prx3), was upregulated also in the cerebellar tissue lysates prepared from unchallenged stefin B-deficient mice, while after LPS challenge Prx3 was upregulated in stefin B-deficient brain and cerebella. Our results indicate the important role of oxidative stress in the progression of the disease. In addition, we also performed studies on the structure and folding of stefin B, which has been used as a model protein. In the last year we have continued to study the role of polyphenolic anti-oxidants on protein aggregation and have observed the synergy of the inhibitory action of some polyphenols including curcumin and quercetin and vitamin C (vitC) (Jahić Mujkić et al., 2021, Antioxidants). In an overview, E. Žerovnik (Žerovnik E, 2021, Front. Chem.) discussed the common traits between amyloid-forming proteins and known pore-forming proteins, including viroporins. Further along these lines together with collaborators from NIC (prof. Novič's group) we have searched machine-learning methods to predict the transmembrane regions and ganglioside binding sites in amyloid-forming proteins (Venko et al., 2021, Front. Mol. Neurosci). A set of 30 amyloid-forming proteins was used as the database. A range of amino-acid sequence tools were then applied, in order to predict AP domains and provide context on future experiments that are needed to contribute towards a deeper understanding of amyloid toxicity, which fitted well with the known experimental data.

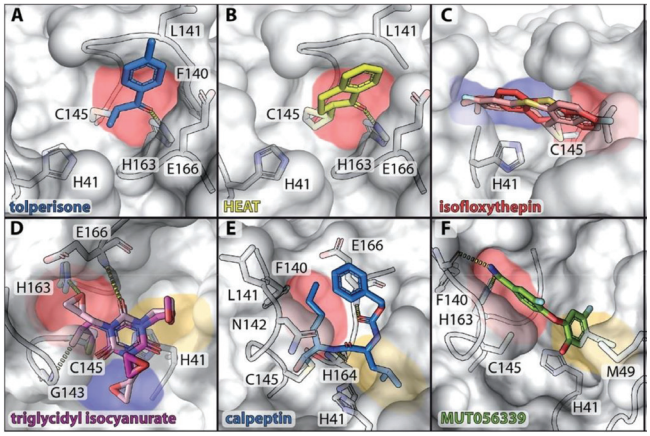

*Figure 2: Covalent and non-covalent binders in the active site of M^{pro}. Bound compounds are depicted as coloured sticks while the surface of M^{pro} is shown in grey with selected interacting residues as sticks. Substrate binding pockets are coloured as in Figure 1. Hydrogen bonds are depicted by dashed lines. (***A***) tolperisone, (***B***) HEAT, (***C***) isofloxythepin, (***D***) triglycidyl isocyanurate, (***E***) calpeptin, (***F***) MUT056399.*

The crucial work of our department was undoubtedly a huge collaboration on understanding the molecular features of the SARS-CoV-2 main protease, a key drug target of the virus as one of the two viral proteases essential to yield functional viral proteins (Günther et al., Turk D., 2021) published in Science. Screening of more than 5000 compounds that are either approved drugs or drugs in clinical trials resulted in several crystal structures of the active site and allosteric inhibitors of the SARS-CoV-2 main protease. The most potent inhibitor, calpeptin, binds covalently in the active site, whereas the second-most potent, pelitinib, binds at an allosteric site. Calpeptin dual targeting of cathepsins and Mpro is also explored as an important path for the therapeutical inhibition of SARS-CoV-2. This was also the very first work published on SARS-CoV-2 virus from Slovenia.

Tušar et al. (2021, Int J Mol Sci) wrote a review analysing the mechanisms of inhibition of cysteine proteases on the basis of structural information and compiled kinetic data. It reveals that the protein fold is not a major obstacle to the evolution of a protease inhibitor and that there appears to be no general rule governing the inhibitory mechanism. However, the analysis suggests that the shape of the active site cleft of proteases imposes some restraints, mainly based on the shape and solvent exposure of the S1 binding site. While the pocket-shaped S1 binding site buried in the structure of the protease enables substrate-like binding mechanisms of inhibitors, with the S1 binding site in part exposed to a solvent, the substrate-like inhibition cannot be employed. Except for papain-like proteases, all proteases appear to belong to the first group of proteases.

The novel finding that cathepsin X is a dimeric protein (Dolenc et al., 2021, BBA Proteins Proteom.) opens new horizons in the understanding of its function and the underlying pathophysiological mechanisms of various diseases, including neurodegenerative disorders in humans. Namely, human cathepsin X is an exopeptidase belonging to the cathepsin family of 11 lysosomal cysteine proteases. We expressed recombinant procathepsin X in *Pichia pastoris* and *in vitro* cleaved it into its active, mature form using aspartic cathepsin E. We found, using size-exclusion chromatography, X-ray crystallography, and small-angle X-ray scattering, that cathepsin X is biologically active as a homodimer with a molecular weight of ~53 kDa.

In addition, we collaborated with other research groups from Slovenia and several foreign countries (Germany, Austria, Hungary, Switzerland, Croatia, Poland, United Kingdom, Netherlands, Japan and USA).

Some outstanding publications in the past year

1. Biasizzo M, Trstenjak-Prebanda M, Dolinar K, Pirkmajer S, Završnik J, Turk B, Kopitar-Jerala N. Cystatin C Deficiency Increases LPS-Induced Sepsis and NLRP3 Inflammasome Activation in Mice. Cells. 2021. doi: 10.3390/cells10082071.
2. Jahić Mujkić A, Tušek Žnidarič M, Berbić S, Žerovnik E. Synergy of the Inhibitory Action of Polyphenols Plus Vitamin C on Amyloid Fibril Formation: Case Study of Human Stefin B. Antioxidants (Basel). 2021. doi: 10.3390/antiox10091471.
3. Bernegger S, Vidmar R, Fonovic M, Posselt G, Turk B, Wessler S. Identification of Desmoglein-2 as a novel target of Helicobacter pylori HtrA in epithelial cells. Cell Commun Signal. 2021. doi: 10.1186/s12964-021-00788-x.
4. Günther et al., X-ray screening identifies active site and allosteric inhibitors of SARS-CoV-2 main protease. Science. 2021. doi: 10.1126/science.abf7945.
5. Dolenc I, Štefe I, Turk D, Taler-Verčič A, Turk B, Turk V, Stoka V. Human cathepsin X/Z is a biologically active homodimer. Biochim Biophys Acta Proteins Proteom. 2021. doi: 10.1016/j.bbapap.2020.140567.

Awards and Appointments

1. Monika Biasizzo: Young Investigator Award Nomination: Best Speaker at 38th Winter School on proteinases and their inhibitors, ASBMB virtual conference, Impaired autophagy and increased susceptibility to LPS-induced sepsis in cystatin C-deficient mice.
2. Eva Vidak: Young Investigator Award Nomination: Best Speaker at 38th Winter School on proteinases and their inhibitors, ASBMB virtual conference, Identification of extracellular substrates of caspases 3, 7, and 1.

Organization of conferences, congresses and meetings

1. 38th Winter School on proteinases and their inhibitors, ASBMB virtual conference, 24– 26 February 2021 (virtual), co-organiser.

Patent granted

1. Stephen James Moore, Margaret Thy Luu Hyugen, Daniel Robert Hostetter, Olga Vasiljeva, Jason Gary Sagert, Jonathan Alexander Terrett, James William West, Matrix metalloprotease-cleavable and serine protease-cleavable substrates and methods of use thereof, US11046759 (B2), US Patent Office, 29. 06. 2021.

INTERNATIONAL PROJECTS

1. EFSA - EU-FORA; Implementation of Matrix Effects into Chemical Food Contaminant Risk Assessment
   Asst. Prof. Livija Tušar
   European Food Safety Authority - EFSA
2. The Role of Cystatins in Neuroinflamamtion
   Prof. Nataša Kopitar – Jerala
   Slovenian Research Agency
3. Innovative ECO plasma seed treatment (for sowing and for human and animal diet/ nutrition
   Prof. Boris Turk
   Ministry of Education, Science and Sport

RESEARCH PROGRAMMES

1. Structural biology
   Prof. Dušan Turk
2. Proteolysis and its regulation
   Prof. Boris Turk

R & D GRANTS AND CONTRACTS

1. Molecular genetic biomarkers and mechanisms of unresponsiveness to biological therapyanti-TNF in patients with chronic immune diseases
   Prof. Boris Turk
2. Structural insight into the mechanism of Clostridium difficle surface formation
   Prof. Dušan Turk
3. Cathepsins B and X in breast cancer stem cells – molecular targets and relevance for antitumor therapy
   Prof. Marko Fonović
4. Aptamers and hydrodynamic cavitation, an accessible tool for the analysis of organic residuals in archaeological pottery
   Prof. Marko Fonović
5. Role of legumain in infection and inflammation
   Prof. Marko Fonović
6. Role of cysteine cathepsins in complement activation in cancer
   Prof. Boris Turk
7. Mineral inclusions in garnet from macroscopic to atomic scale: Opening the petrogenetic archive
   Prof. Boris Turk
8. Cathepsin-based non-invasive diagnostics and theranostics of cancer
   Prof. Boris Turk
9. Human cathepsin F: An unusual cysteine protease involved in neurodegeneration
   Prof. Veronika Stoka
10. Dissecting cancer activome to develop new generation of antibody-drug conjugates
    Prof. Boris Turk
11. Systemic determination of legumain physiological roles
    Prof. Marko Fonović

NEW CONTRACTS

1. Determination of protein concentration by Bradford methodology
   Prof. Marko Fonović
   Lek d. d.
2. Proteomic analysis
   Prof. Marko Fonović

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BIBLIOGRAPHY

Original Article

1. Mojca Trstenjak-Prebanda, Petra Matjan-Štefin, Boris Turk, Nataša Kopitar-Jerala, "Altered expression of peroxiredoxins in mouse model of progressive myoclonus epilepsy upon LPS-induced neuroinflammation", *Antioxidants*, 2021, **10**, 3, 357.
2. Alma Jahić Mujkić, Magda Tušek-Žnidarič, Selma Berbić, Eva Žerovnik, "Synergy of the inhibitory action of polyphenols plus vitamin C on amyloid fibril formation: case study of human stefin B", *Antioxidants*, 2021, **10**, 9, 1471.
3. Iztok Dolenc, Ivica Klemenčič, Dušan Turk, Ajda Taler-Verčič, Boris Turk, Vito Turk, Veronika Stoka, "Human cathepsin X/Z is a biologically active homodimer", *Biochimica et biophysica acta. Proteins and proteomics.*, 2021, **1869**, 2, 140567.
4. Hikmat H. Assi *et al.* (27 authors), "Conditional PD-1/PD-L1 probody therapeutics induce comparable antitumor immunity but reduced systemic toxicity compared with traditional anti–PD-1/PD-L1 agents", *Cancer immunology research*, 2021, **9**, 12, 1451-1464.
5. Csaba András Schvarcz *et al.* (14 authors), "Modulated electro-hyperthermia induces a prominent local stress response and growth inhibition in mouse breast cancer isografts", *Cancers*, 2021, **13**, 7, 1744.
6. Sabine Bernegger, Robert Vidmar, Marko Fonović, Gernot Posselt, Boris Turk, Silja Wessler, "Identification of Desmoglein-2 as a novel target of Helicobacter pylori HtrA in epithelial cells", *Cell communication and signaling*, 2021, **19**, 1, 108.
7. Monika Biasizzo, Mojca Trstenjak-Prebanda, Klemen Dolinar, Sergej Pirkmajer, Janja Završnik, Boris Turk, Nataša Kopitar-Jerala, "Cystatin C deficiency increases LPS-induced sepsis and NLRP3 inflammasome activation in mice", *Cells*, 2021, **10**, 8, 2071.
8. Laura Kist de Ruijter *et al.* (14 authors), "First-in-human study of the biodistribution and pharmacokinetics of $^{89}Zr-CX-072$, a novel immunopet tracer based on an anti-PD-L1 probody", *Clinical cancer research*, 2021, **27**, 19, 5325-5333.
9. Katja Venko, Marjana Novič, Veronika Stoka, Eva Žerovnik, "Prediction of transmembrane regions, cholesterol and ganglioside binding sites in amyloid-forming proteins indicate potential for amyloid pore formation", *Frontiers in molecular neuroscience*, 2021, **14**, 619496.
10. Jure Pražnikar, "Scaling laws of graphs of 3D protein structures", *Journal of bioinformatics and computational biology*, 2021, **19**, 1, 2050050.
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12. Julius Kostan *et al.* (17 authors), "Molecular basis of F-actin regulation and sarcomere assembly via myotilin", *PLoS biology*, 2021, **19**, 4, e3001148.
13. Iñaki Etxeberria *et al.* (24 authors), "Antitumor efficacy and reduced toxicity using an anti-CD137 Probody therapeutic", *Proceedings of the National Academy of Sciences of the United States of America*, 2021, **118**, 26, e2025930118.
14. Marko Fonović, Tamara Leskovar, Iztok Štamfelj, "Določitev spola na podlagi spolno dimorfnih amelogeninskih peptidov v človeški zobni sklenini", *Revija za kriminalistiko in kriminologijo*, 2021, **72**, 2, 117-128.
15. Sebastian Günther *et al.* (102 authors), "X-ray screening identifies active site and allosteric inhibitors of SARS-CoV-2 main protease", *Science*, 2021, **372**, 6542, 642-646.

Review Article

1. Daniel J. Klionsky *et al.* (2299 authors), "Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)", *Autophagy*, 2021, **17**, 1, 1-382.
2. Bernarda Majc, Metka Novak, Nataša Kopitar-Jerala, Anahid Jewett, Barbara Breznik, "Immunotherapy of glioblastoma: current strategies and challenges in tumor model development", *Cells*, 2021, **10**, 2, 265.
3. Eva Žerovnik, "Viroporins vs. other pore-forming proteins: what lessons can we take", *Frontiers in chemistry*, 2021, **9**, 626059.
4. Livija Tušar, Aleksandra Usenik, Boris Turk, Dušan Turk, "Mechanisms applied by protein inhibitors to inhibit cysteine proteases", *International journal of molecular sciences*, 2021, **22**, 3, 997.
5. Eva Žerovnik, "On similarities and differences of HIV and SARS Cov-2: open questions relevant for COVID-19 diseas", *Journal biotechnology and biomedicine*, 2021, **4**, 4, 147-158.

Short Article

1. Dušan Turk, Gregor Gunčar, "Thyroxine hormones visualized by the cryo-EM structure of bovine thyroglobulin", *Acta crystallographica. Section D, Structural biology,* 2021, **77**, 1346-1347.

Independent Component Part or a Chapter in a Monograph

1. Olga Vasiljeva, Lisa Sevenich, Thomas Reinheckel, "Analyzing the role of proteases in breast cancer progression and metastasis using primary cells from transgenic oncomice", In: *Metastasis: methods and protocols*, (Methods in molecular biology **2294**), Humana Press, 2021, 275-293.
2. Alexey A. Tsukanov, Olga Vasiljeva, "Nanomaterials interaction with cell membranes: computer simulation studies", In: *Multiscale biomechanics and tribology of inorganic and organic systems*, (Springer tracts in mechanical engineering), Springer, 2021, 189-210.

Patent Application

1. Olga Vasiljeva, Emma Geertruida Elisabeth De Vries, Marjolijn N. Lubde Hooge, Annelies Jorritsma-Smit, Martin Pool, Danique Giesen, Iris Kok, Linda N. Broer, Mark Stroh, *Positron emission tomography imaging of activatable binding polypeptides and related compositions thereof*, US2020405890 (A1), US Patent Office, 31. 12. 2020.
2. Olga Vasiljeva, Stephen James Moore, Bruce Howng, Susan K. Lyman, Luc Roland Desnoyers, *Methods of qualitatively and/or quantitatively analyzing properties of activatable antibodies and uses thereof*, US2021025877 (A1), US Patent Office, 28. 01. 2021.

Patent

1. Stephen James Moore, Margaret Thy Luu Hyugen, Daniel Robert Hostetter, Olga Vasiljeva, Jason Gary Sagert, Jonathan Alexander Terrett, James William West, *Matrix metalloprotease-cleavable and serine protease-cleavable substrates and methods of use thereof*, US11046759 (B2), US Patent Office, 29. 06. 2021.

Theses and Mentoring

1. Samra Hasanbašić, *Investigating the influence of intrinsic and extrinsic factors on amyloid fibrillation: a study on stefin B model:* doctoral dissertation, Tuzla, 2021 (mentor Eva Žerovnik).
2. Alma Mujkić, *Influence of antioxidants on amyloid fibrillation; in vitro studies on the model of human stefin B and cystatin:* doctoral dissertation, Tuzla, 2021 (mentor Eva Žerovnik).