DEPARTMENT OF BIOCHEMISTRY, MOLECULAR AND STRUCTURAL BIOLOGY B-1

The research activities of the members of the department are largely focused on studies of the physiological role of proteases and their endogenous protein inhibitors in normal and pathological conditions, the mechanism of their action and regulation, as well as their properties and structure. As molecular mechanisms of protease action are only partially understood, since only a limited number of physiological substrates of a few proteases has been identified, a lot of work 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, cancer, cardiovascular, neurologic and neurodegenerative disorders. Thus, proteases represent an extremely important group of targets for therapeutic intervention.

In cancer studies we have shown that L-leucyl-leucine methyl ester (LLOMe), a lysosomotropic detergent, influences cell death and this depends on the levels of cathepsin C. We have shown that the deletion of the endogenous cathepsin inhibitor stefin B results in sensitizing primary murine breast-cancer cells to cell death without affecting the release of cathepsins, whereas the simultaneous ablation of cathepsins B and L largely protects mouse embryonic fibroblasts against cell death. However, due to the extreme sensitivity of monocytes to LLOMe, it appears that the drug may not be suitable for anticancer therapy due to a risk of systemic toxicity (Kavčič et al., 2020).

As we know from animal models cancer studies, the specific inhibitory affinity of proteases can be utilized for targeted drug delivery. We confirmed before the selective targeting of cathepsins by stefin A-conjugated liposomes *in vitro* and *in vivo* and demonstrated the potential of this approach for cancer diagnostics and treatment. In the last year we summarized our studies in a review paper (Vizovišek et al., 2020), where we gave expert opinion on the potential of cysteine cathepsins as therapeutic targets in inflammation-associated diseases. We described the direct targeting of cathepsins for treatment purposes and their indirect use in diagnostics. The targeting of cysteine cathepsins has not translated into the clinic; this failure is attributed to off- and on-target side effects and/or the lack of companion biomarkers. This field now embraces developments in diagnostic imaging, the activation of prodrugs and antibody-drug conjugates for targeted drug delivery. The future lies in improved molecular tools and therapeutic concepts that will find a wide spectrum of uses in diagnostic and therapeutic applications.

In the continuation of our protease-specificity studies, we performed the proteomic characterization of cysteine proteases gingipains from the bacteria P. gingivalis, causing periodontitis (Hočevar et al., 2020). HRgpA and RgpB gingipains have Arg-specificity, while Kgp gingipain is Lys-specific. Together they can cleave an array of proteins and importantly contribute to the development of periodontitis. We focused on gingipain-exerted proteolysis at the cell surface of human gingival epithelial cells [telomerase immortalized gingival keratinocytes (TIGK)] in order to better understand the molecular mechanisms behind tissue destruction in this disease of the gums. Using mass spectrometry, we investigated the whole sheddome/degradome of TIGK cell surface proteins by *P. gingivalis* strains differing in gingipain expression and by purified gingipains, and performed the first global proteomic analysis of gingipain proteolysis at the membrane. Most of the identified gingipain substrates were molecules involved in adhesion, suggesting that gingipains can cause tissue damage through the cleavage of cell contacts, resulting in cell detachment and rounding, and consequently leading to anoikis.

In a review paper (Biasizzo and Kopitar Jerala, 2020) the interplay between NLRP3 Inflammasome and autophagy is discussed. Namely, the NLRP3 inflammasome is a cytosolic multi-protein complex that induces inflammation and pyroptotic cell death in response to both pathogen and endogenous activators, leading to the formation of the inflammasome complex, which results in the activation of caspase-1, followed by the cleavage and release of pro-inflammatory cytokines. The excessive activation of NLRP3 inflammasome can contribute to the development of inflammatory diseases and cancer. Autophagy is a vital intracellular process for recycling and the removal of damaged proteins and organelles, as well as the destruction of intracellular pathogens. The autophagy dysfunction can lead to diseases with hyperinflammation and excessive activation of NLRP3 inflammasome and thus acts as a major regulator of inflammasomes. Autophagic removal of NLRP3 inflammasome activators, or NLRP3 inflammasome components, and cytokines can reduce inflammasome activation.

In collaboration with Joel Selkrig and Athanasios Typas, EMBL Heidelberg, Germany, we reported on the trafficking of lysosomal cysteine cathepsins to the extracellular space and to the nucleus upon infection with *Salmonella enterica Typhimurium*. Typas' lab used a proteomics approach to selectively quantify newly synthesized host proteins during infection. Nuclear cathepsin activity was required for pyroptotic cell death via the non-canonical inflammasome activation. Nuclear targeting of a cathepsin inhibitor, stefin B or pharmacological cathepsin inhibition suppressed *S. enterica* Typhimurium-induced cell death. Moreover, cathepsin inhibition reduced the expression of gasdermin D, a key protein in pyroptotic cell death (Sekrig et al., 2020).

Apart from cysteine cathepsins, several aspects of their endogenous inhibitors, stefins and cystatins are studied by our group. For example, stefins are being used as model proteins to study protein folding and aggregation. We have determined the effect of polyphenols and vitamin C and some other anti-oxidants on protein aggregation (Jahić et al., 2020). Polyphenols, such as curcumin, are mostly inhibiting protein aggregation, which maybe explains their neuroprotective role as protein misfolding and aggregation are involved in neurodegenerative diseases. We are using the amyloidogenic protein stefin B as a prototype of amyloidogenic proteins and explore its pore formation, for which we have started collaboration with the group of biophysics (Štrancar's lab) and hope to see protein oligomers in interaction with cellular membranes.

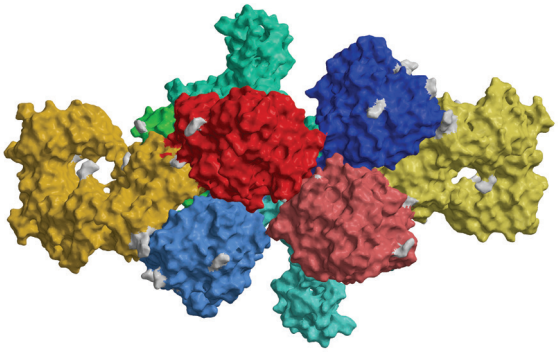

Figure 1: Thyroglobulin is a dimer, formed by two polypeptide chains. The surface representations shows the structure colored according to the structure divided in regions: N-terminal domain (blue), core (cyan), flap (green), arm (yellow), C-terminal domain (red). To differentiate the polypeptide chains, one is shown with dimmed colors.

The most important work that resulted in the determination of the 3D structure of human thyroglobulin, the protein precursor of thyroid hormones, which are essential for growth, development and the control of metabolism in vertebrates, was done in collaboration with the group of Jan Loewe from MRC Cambridge (UK) (Coscia et al., 2020). The structure of full-length human thyroglobulin at a resolution of approximately 3.5 Å was determined by cryo-electron microscopy. We identified all of the hormonogenic tyrosine pairs in the structure, and verified them using site-directed mutagenesis and in-vitro hormone-production assays using human thyroglobulin expressed in HEK293T cells. Our analysis revealed that the proximity, flexibility and solvent exposure of the tyrosines are the key characteristics of hormonogenic sites. We transferred the reaction sites from thyroglobulin to an engineered tyrosine donor-acceptor pair in the unrelated bacterial maltose-binding protein, which yielded hormone production with an efficiency comparable to that of thyroglobulin. Our study thus provides a framework to further understand the production and regulation of thyroid hormones.

In addition, the group for structural biology determined several structures of bacterial enzymes that can contribute to the development of novel antibacterial drugs. In pursuit of novel drug targets for the human pathogen S. aureus, we thus studied peptidoglycan N-acetylglucosaminidases, whose structures are composed of two domains forming a V-shaped active site cleft. Combined insights from crystal structures supported by site-directed mutagenesis, modelling, and molecular dynamics enabled us to elucidate the substrate-binding mechanism of SagB and AtlA-gl. This mechanism requires domain sliding from the open form observed in their crystal structures, leading to polysaccharide substrate binding in the closed form, which can enzymatically process the bound substrate. We suggest that these two hydrolases must exhibit unusual extents of flexibility to cleave the rigid structure of a bacterial cell wall (Pintar et al., 2020). The second target was SecA protein, a major component of the general bacterial secretory system. It is an ATPase that couples nucleotide hydrolysis to protein translocation. In some Gram-positive pathogens, a second paralogue, SecA2, exports a different set of substrates, usually virulence factors. To identify SecA2 features different from SecA(1)s, we determined the crystal structure of SecA2 from Clostridioides difficile, an important nosocomial pathogen, in apo and ATP-γ-S-bound form (Lindič et al., 2020).

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

Some outstanding publications in the past year

1. Kavčič, N, Butinar, M, Sobotič, B, Hafner Česen, M, Petelin, A, Bojič, L, Zavašnik-Bergant, T, Bratovš, A, Reinheckel, T, Turk, B. Intracellular cathepsin C levels determine sensitivity of cells to leucyl-leucine methyl ester-triggered apoptosis. (2020) *FEBS journal*, 287(23):5148-5166.
2. Selkrig J, Li N, Hausmann A, Mangan MSJ, Zietek M, Mateus A, Bobonis J, Sueki A, Imamura H, El Debs B, Sigismondo G, Florea BI, Overkleeft HS, Kopitar-Jerala N, Turk B, Beltrao P, Savitski MM, Latz E, Hardt WD, Krijgsveld J, Typas A. Spatiotemporal proteomics uncovers cathepsin-dependent macrophage cell death during Salmonella infection. (2020), Nature Microbiology, vol. 5. 1119–1133.
3. Coscia, Francesca, Taler-Verčič, Ajda, Chang, Veronica T., Sinn, Ludwig, O'reilly, Francis J., Izoré, Thierry, Renko, Miha, Berger, Imre, Rappsilber, Juri, Turk, Dušan, Löwe, Jan. The structure of human thyroglobulin. (2020) Nature, 578, 627-630.

Patents granted

1. Stephen James Moore, Margaret Thy Luu Nguyen, Daniel Robert Hostetter, Olga Vasiljeva, Jeanne Grace Flandez, Substrates of matrix metalloproteinase and other cleavable moieties and methods of use thereof, AU2014324884 (B2), Australian Patent Office, 26. 03. 2020.
2. James William West, Li Mei, Stephen James Moore, Margaret Thy Luu Nguyen, Daniel Robert Hostetter, Olga Vasiljeva, Jason Sagert, Jonathan Terrett, Anti-PDL1 antibodies, activatable anti-PDL1 antibodies, and methods of use thereof, US10669339 (B2), US Patent Office, 02. 06. 2020.

INTERNATIONAL PROJECTS

1. Supply of DPPI Enzyme and the Non-exclusive License Rights
   Prof. Dušan Turk
   Prozymex A/s
2. COST CA 15203; Mitochondrial Mapping: Evolution-Age-Gender-Lifestyle-Environment
   Prof. Nataša Kopitar – Jerala
   Cost Office
3. COST CA15124; NEUBIAS - A New Network of European Bioimage Analysts to Advance Life Science Imaging
   Asst. Prof. Tina Zavašnik Bergant
   Cost Office
4. COST CA15214; An Integrative Action for Multidisciplinary Studies on Cellular Structural Networks
   Prof. Nataša Kopitar – Jerala
   Cost Office
5. The Role of Cystatins in Neuroinflamamtion
   Prof. Nataša Kopitar – Jerala
   Slovenian Research Agency

RESEARCH PROGRAMMES

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

R & D GRANTS AND CONTRACTS

1. Cathepsin X inhibitors impair the resistence of tumor cells to antiprotease therapy
   Prof. Boris Turk
2. Molecular genetic biomarkers and mechanisms of unresponsiveness to biological therapyanti-TNF in patients with chronic immune diseases
   Prof. Boris Turk
3. Structural insight into the mechanism of Clostridium difficle surface formation
   Prof. Dušan Turk
4. Inhibition of Staphylococcus aureus cell wall remodeling
   Prof. Dušan Turk
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. Innovative ECO plasma seed treatment (for sowing and for human and animal diet/ nutrition
    Prof. Boris Turk
    Ministry of Education, Science and Sport
11. Reimbursement of costs of scientific publications in golden open access for 2019, 2020
    Prof. Boris Turk
    Slovenian Research Agency
12. Lyposomal Proteases in Semaphorin Signaling and Cell Polarity
    Prof. Boris Turk
    Icgeb - International Centre For Genetic

NEW CONTRACT

1. Collaboration on quantification of cellular proteins by LC-MS/MS based proteomic analysis
   Prof. Marko Fonović
   Lek d. d.

VISITORS FROM ABROAD

1. Nora Diéguez Martínez, Barcelona University (UAB), Barcelona, Spain, 13 January to 14 March 2020
2. Gian Pietro Pietri, University of Rijeka, Croatia, 3-10 October 2020

STAFF

Researchers

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15. Dr. Nataša Lindič
16. Dr. Georgy Mikhaylov
17. *Dr. Sara Pintar, left 01.08.20*
18. Dr. Jure Pražnikar*
19. *Dr. Vida Puizdar, retired 16.01.20*
20. *Dr. Ajda Taler-Verčič*, left 01.10.20*
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Postgraduates

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38. Maja Orehek, B. Sc.
39. Dejan Pelko
40. Polonca Pirš

Note:
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Original Article

1. Miha Bahun, Marko Šnajder, Dušan Turk, Nataša Poklar Ulrih, "Insights into the maturation of pernisine, a subtilisin-like protease from the hyperthermophilic archaeon *Aeropyrum pernix*", *Applied and environmental microbiology*, 2020, **86**, 17, e00971-20.
2. Magali Humbert *et al.* (18 authors), "Assessing autophagy in archived tissue or how to capture autophagic flux from a tissue snapshot", *Biology*, 2020, **9**, 3, 59.
3. Danique Giesen, Linda N. Broer, Marjolijn N. Lub-de Hooge, Irina Popova, Bruce Howng, Margaret Nguyen, Olga Vasiljeva, Elisabeth G. de Vries, Martin Pool, "Probody therapeutic design of 89Zr-CX-072 promotes accumulation in PD-L1-expressing tumors compared to normal murine lymphoid tissue", *Clinical cancer research*, 2020, **26**, 15, 3999-4009.
4. Sara Pintar, Jure Borišek, Aleksandra Usenik, Andrej Perdih, Dušan Turk, "Domain sliding of two *Staphylococcus aureus* N-acetylglucosaminidases enables their substrate-binding prior to its catalysis", *Communications biology*, 2020, **3**, 178.
5. Nežka Kavčič, Miha Butinar, Barbara Sobotič, Maruša Hafner Česen, Ana Petelin, Lea Bojič, Tina Zavašnik-Bergant, Andreja Bratovš, Thomas Reinheckel, Boris Turk, "Intracellular cathepsin C levels determine sensitivity of cells to leucyl-leucine methyl ester-triggered apoptosis", *FEBS journal*, 2020, **287**, 23, 5148-5166.
6. Katarina Hočevar, Matej Vizovišek, Alicia Wong, Joanna Lubos-Kozieł, Marko Fonović, Barbara Potemba, Richard J. Lamont, Jan Potempa, Boris Turk, "Proteolysis of gingival keratinocyte cell surface proteins by gingipains secreted from *Porphyromonas gingivalis* - proteomic insights into mechanisms behind tissue damage in the diseased gingiva", *Frontiers in microbiology*, 2020, **11**, 722.
7. Beáta Róka, Pál Tod, Tamás Kaucsár, Matej Vizovišek, Robert Vidmar, Boris Turk, Marko Fonović, Gábor Szénási, Péter Hamar, "The acute phase response is a prominent renal proteome change in sepsis in mice", *International journal of molecular sciences*, 2020, **21**, 1, 200.
8. Pál Tod, Beáta Róka, Tamás Kaucsár, Krisztina Szatmári, Matej Vizovišek, Robert Vidmar, Marko Fonović, Gábor Szénási, Péter Hamar, "Time-dependent miRNA profile during septic acute kidney injury in mice", *International journal of molecular sciences*, 2020, **21**, 15, 5316.
9. Nataša Lindič, Jure Loboda, Aleksandra Usenik, Robert Vidmar, Dušan Turk, "The structure of *clostridioides difficile* SecA2 ATPase exposes regions responsible for differential target recognition of the SecA1 and SecA2-dependent systems", *International journal of molecular sciences*, 2020, **21**, no 17, 6153.
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11. Aleš Mohorič, Janko Božič, Polona Mrak, Kaja Tušar, Lin Chenyun, Ana Sepe, Urška Mikac, Georgy Mikhaylov, Igor Serša, "*In vivo* continuous three-dimensional magnetic resonance microscopy: a study of metamorphosis in Carniolan worker honey bees *(Apis mellifera carnica*)", *Journal of Experimental Biology*, 2020, **223**, 21, jeb225250.
12. Francesca Coscia, Ajda Taler-Verčič, Veronica T. Chang, Ludwig Sinn, Francis J. O'Reilly, Thierry Izoré, Miha Renko, Imre Berger, Juri Rappsilber, Dušan Turk, Jan Löwe, "The structure of human thyroglobulin", *Nature*, 2020, **578**, 627-630.
13. Joel Selkrig *et al.* (21 authors), "Spatiotemporal proteomics uncovers cathepsin-dependent macrophage cell death during Salmonella infection", *Nature microbiology*, 2020, **5**, 1119–1133.
14. Alma Jahić, Magda Tušek-Žnidarič, Sara Pintar, Selma Berbić, Eva Žerovnik, "The effect of three polyphenols and some other anti-oxidant substances on amyloid fibril formation by human cystatin C", *Neurochemistry International*, 2020, **140**, 104806.
15. Katja Bezek, Ana Petelin, Jure Pražnikar, Esther Nova, Noemi Redondo, Marcos Ascensión, Zala Jenko Pražnikar, "Obesity measures and dietary parameters as predictors of gut microbiota phyla in healthy individuals", *Nutrients*, 2020, **12**, 9, 2695.
16. Olga Vasiljeva, Elizabeth Menendez, Margaret Nguyen, Charles S. Craik, W. Michael Kavanaugh, "Monitoring protease activity in biological tissues using antibody prodrugs as sensing probes", *Scientific reports*, 2020, **10**, 5894.
17. Sabine Bernegger *et al.* (16 authors), "A novel FRET peptide assay reveals efficient *Helicobacter pylori* HtrA inhibition through zinc and copper binding", *Scientific reports*, 2020, **10**, 10563.
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19. Marin Chomet, Maxime Schreurs, Margaret Nguyen, Bruce Howng, Ruth Villanueva, Michael Krimm, Olga Vasiljeva, Guus A. M. S. van Dongen, Danielle J. Vugts, "The tumor targeting performance of anti-CD166 Probody drug conjugate CX-2009 and its parental derivatives as monitored by ^{89}Zr-immuno-PET in xenograft bearing mice", *Theranostics*, 2020, **10**, 13, 5815-5828.

Review Article

1. Bernarda Majc, Tilen Sever, Miki Zarić, Barbara Breznik, Boris Turk, Tamara Lah Turnšek, "Epithelial-to-mesenchymal transition as the driver of changing carcinoma and glioblastoma microenvironment", *Biochimica et biophysica acta. BBA, Molecular cell research*, 2020, **1867**, 10, 118782.
2. Aljaž Gaber, Brigita Lenarčič, Miha Pavšič, "Current view on EpCAM structural biology", *Cells*, 2020, **9**, 6, 1361.
3. Matej Vizovišek, Eva Vidak, Urban Javoršek, Georgy Mikhaylov, Andreja Bratovš, Boris Turk, "Cysteine cathepsins as therapeutic targets in inflammatory diseases", *Expert opinion on therapeutic targets*, 2020, **24**, 6, 573-588.
4. Monika Biasizzo, Nataša Kopitar-Jerala, "Interplay between NLRP3 inflammasome and autophagy", *Frontiers in immunology*, 2020, **11**, 591803.
5. Ajda Taler-Verčič, Marko Goličnik, Aljoša Bavec, "The structure and function of paraoxonase-1 and its comparison to paraoxonase-2 and -3", *Molecules*, 2020, **25**, 24, 5980.

Patent

1. Stephen James Moore, Margaret Thy Luu Nguyen, Daniel Robert Hostetter, Olga Vasiljeva, Jeanne Grace Flandez, *Substrates of matrix metalloproteinase and other cleavable moieties and methods of use thereof*, AU2014324884 (B2), Australian Patent Office, 26. 03. 2020.
2. James William West, Li Mei, Stephen James Moore, Margaret Thy Luu Nguyen, Daniel Robert Hostetter, Olga Vasiljeva, Jason Sagert, Jonathan Terrett, *Anti-PDL1 antibodies, activatable anti-PDL1 antibodies, and methods of use thereof*, US10669339 (B2), US Patent Office, 02. 06. 2020.

Theses and Mentoring

1. Andreja Bratovš, *Targeting the tumor microenvironment with nanoparticles for treatment and diagnostics:* doctoral dissertation, Ljubljana, 2020 (mentor Olga Vasiljeva; co-mentor Boris Turk).
2. Katarina Hočevar, *Role of extracellular cysteine proteases in the processing of membrane proteins:* doctoral dissertation, Ljubljana, 2020 (mentor Boris Turk).