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 in normal and pathological conditions, the mechanism of their action and regulation, as well as their properties and structure. Part of the activities is devoted to the development of tools that allow us to understand the properties of proteases and other enzymes, as well as to enable their monitoring and manipulation in in-vivo conditions.

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 this regulation can lead to pathologies such as autoimmune, neurological and cardiovascular disorders, cancer and osteoporosis. However, protease signalling pathways are only partially understood. Currently, only a minor subset of physiological substrates for a limited number of proteases has been identified, and their physiological regulation is still not well understood.

As one of the leaders in the field, we were invited to write a review paper in one of the most important journals in the field, Trends in Biochemical Sciences. In this article we gave an overview of the progress and current trends in the field of proteases and their application to biomarker discovery and in-vivo imaging.

We have continued with proteomic approaches devoted to the identification of protease specificities and the identification of physiological protease substrates, as well as with small-molecule approaches in order to develop novel activity-based probes. In collaboration with dr. M. Drag (University of Wroclaw), we used a hybrid combinatorial substrate library (HyCoSuL) approach to obtain specific fluorogenic substrates and an activity-based probe for monitoring cathepsin L activity in the breast-cancer cell line MDA-MB-231. The use of this probe enabled us to distinguish the activity of cathepsin L from that of other cathepsins, particularly cathepsin B, which is abundant and ubiquitously expressed in normal and transformed cell types. Overall, these studies demonstrate that HyCoSuL-derived small-molecule probes are valuable tools to image cathepsin L activity in living cells. This approach thus enables the evaluation of the cathepsin L function in tumorigenesis and is applicable to other cysteine cathepsins.

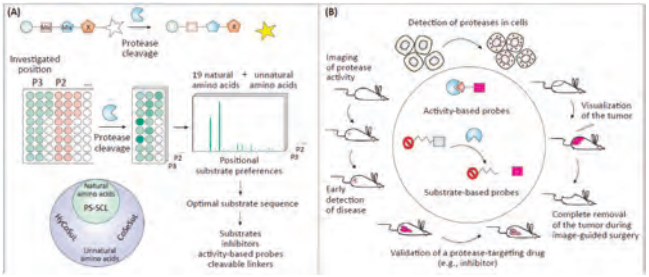

Figure 1. A) Chemical tools for protease profiling. B) In-vivo imaging modalities for laboratory animals.

Besides mammalian proteases we have focused on bacterial proteases, namely, gingipains. Gingipains are extracellular cysteine proteases of the oral pathogen *Porphyromonas gingivalis* and its most potent virulence factors. They can degrade a great variety of host proteins, thereby helping the bacterium to evade the host immune response, deregulate signalling pathways, trigger anoikis and, finally, cause tissue destruction. Host cell-surface proteins targeted by gingipains are the key and span three groups of substrates: immune-regulatory proteins, signalling pathways regulators and adhesion molecules. The analysis revealed that gingipains predominantly inactivate their substrates by cleaving them at one or more sites, or through complete degradation. Sometimes, gingipains were even found to initially shed their membrane substrates, but this was mostly just the first step in the degradation of the cell-surface proteins.

In addition, we focused on other bacterial enzymes due to the increasing emergence of antibiotic-resistant strains such as MRSA and VRSA. Therefore, additional pathways essential for the survival of bacteria needed to be explored in order to develop new strategies and new classes of antibiotics. We proposed that Autolysin E (AtlE), from Staphylococcus aureus, is a cell-wall-degrading enzyme that is a potential drug target. It is a member of the glycoside hydrolase (GH) class, enzymes that commonly have either two catalytic residues and hydrolyse their substrates by inverting or retaining mechanisms or one catalytic residue and undergo retaining, substrate-assisted catalysis. Using site-directed mutagenesis studies we have identified Glu138 as the only catalytic residue. Quantum mechanics/molecular mechanics (QM/MM) simulations of the possible reaction pathways suggest that hydrolysis proceeds via a retaining, water-assisted mechanism and an oxocarbenium ion-like transition state. Our results, on the basis of data from a member of the hydrolase GH73 family, support the hypothesis of the presence of an alterna-

tive catalytic mechanism in glycoside hydrolases, which can be considered in the design of future AtlE inhibitors. Since we determined the crystal structure of AtlE from *Staphylococcus aureus*, we further used a combination of pharmacophore modelling, similarity search and molecular docking, and identified a series of (Phenylureido)piperidinyl benzamides as potential binders. Surface plasmon resonance (SPR) and saturation-transfer difference (STD) NMR experiments revealed that the selected compounds bind to AtlE in a lower micromolar range. (Phenylureido) piperidinyl benzamides are thus the first reported non-substrate-like compounds that interact with this enzyme and enable the further study of the interaction of small molecules with bacterial AtlE as potential inhibitors of this target.

Another area where we made major progress was the development of drug-delivery systems for cancer treatment. Nanomaterials have become a focus of multidisciplinary research efforts due to their unique physicochemical properties. This includes investigations of their interactions with tumour cells and the stromal compartment of the tumour micro-environment (TME) towards the development of next-generation anticancer therapies. In this work we synthesized aluminium hydroxide mesoporous nanostructures using a modified synthesis method that allowed us to produce a very pure material, without any salt or Al^{3+} cations contaminations. The latter is particularly important, since Al^{3+} can promote the generation of reactive oxygen radicals and subsequent oxidative damage that could lead to toxicity effects. We have shown that agglomerates of radially assembled Al hydroxide crumpled nanosheets exhibit anti-cancer activity due to their selective adsorption properties and positive charge. This effect was demonstrated in vitro by the decreased proliferation and viability of tumour cells, and further confirmed in vivo in two murine cancer models. Moreover, Al hydroxide nanosheets almost completely inhibited the growth of murine melanoma in vivo in combination with a minimally effective dose of doxorubicin. Our direct molecular dynamics simulation demonstrated that Al hydroxide nanosheets can cause a significant ion imbalance in the living cell perimembranous space through the selective adsorption of extracellular anionic species. This approach to TME dysregulation could lay the foundation for the development of novel anti-cancer therapy strategies.

Part of the work was also devoted to studies related to oxidative stress-induced cell-death mechanisms, which are also relevant for cancer. We have studied the protective effects of four different ROS scavengers, N-acetyl-L-cysteine (NAC), α-tocopherol and two superoxide dismutase mimetics, n(III)tetrakis(4-benzoic acid)porphyrin chloride, and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tempol), on cell death induced by oxidative stress. Four different cell-death models, including menadione-triggered necrosis, staurosporine-induced apoptosis and tumour necrosis factor (TNF)-induced apoptosis and necroptosis, were selected to address this question. We have discovered that menadione-triggered necrosis was completely prevented by the classic ROS scavenger NAC and to a substantial amount by the other scavengers, whereas ROS targeting was found to have a marginal effect on the other cell-death modalities investigated. Despite its side effects at higher concentrations, Tempol was able to substantially prevent TNF-induced apoptosis and to a somewhat lesser extent TNF-induced necroptosis. This is also in agreement with previous suggestions that the use of anti-oxidants cannot completely prevent cell death because ROS are not the only trigger of cell death.

It is worth mentioning that our department has, partially through the help of the Center of Excellence Center for Integrative approaches for Chemistry and Biology of Proteins (CIPKEBIP), which we coordinate, established several technological platforms that are all unique in Slovenia and include a structural biology platform, a proteomics platform and a whole-body imaging platform, based on an IVIS Spectrum imaging system and a Quantum FX micro CT. All three platforms are open for external collaborations and several studies resulting from these collaborations have already been published.

In addition, there are numerous other international collaborations with excellent research teams from different countries, including Belgium, Spain, France (through a CEA project), Germany, Sweden, Switzerland, UK, USA, Australia, Hungary and Japan, which resulted in several joint publications.

In addition, we organized a FEBS Workshop on Proteases, Inhibitors and Biological Control in Portorož, which attracts a number of world-class scientists and has become one of the best-known protease-related meetings worldwide. Several members of the department were also invited to give lectures at other international symposia and foreign universities.

Some outstanding publications in the past year

1. Borišek, Jure, Pintar, Sara, Ogrizek, Mitja, Turk, Dušan, Perdih, Andrej, Novič, Marjana. A water-assisted catalytic mechanism in glycoside hydrolases demonstrated on the Staphylococcus aureus autolysin E. *ACS catalysis.* 2018, vol. 8, no. 5, str. 4334-4345, doi: 10.1021/acscatal.8b01064.
2. Lerner, Marat I., Mikhaylov, Georgy, Tsukanov, Alexey A, Lozhkomoev, Alexandr S, Gutmanas, Elazar, Gotman, Irena, Bratovš, Andreja, Turk, Vito, Turk, Boris, Psakhye, Sergey G., Vasiljeva, Olga. Crumpled aluminum hydroxide nanostructures as a microenvironment dysregulation agent for cancer treatment. *Nano letters.* 2018, vol. 18, no. 9, str. 5401-5410, doi: 10.1021/acs.nanolett.8b01592.

3. Vizovišek, Matej, Vidmar, Robert, Drag, Marcin, Fonović, Marko, Salvesen, Guy S., Turk, Boris. Protease specificity : towards in vivo imaging applications and biomarker discovery. TiBS : Trends in biochemical sciences. 2018, vol. 43, no 10, str. 829-844, doi: 10.1016/j.tibs.2018.07.003.

Organisation of conferences, congresses and meetings

1. 35th Winter School on Proteinases and Inhibitors 2018, Tiers, Italy, 28 February to 4 March 2018, co-organizers
2. FEBS Workshop 2018 – Protease, Inhibitors and Biological Control, Portorož, Slovenia, 8-12 September 2018

Patents granted

1. Norbert Schaschke, Olga Vasiljeva, Georgy Mikhaylov, Boris Turk, Cathepsin-binding compound bound to a liposome and its diagnostic and therapeutic use, EP2723387 (B1), European Patent Office, 18. 04. 2018.
2. Sergey Grigorievich Psakhie, Izraillevich Marat Lerner, Elena Alekseevna Glazkova, Olga Vladimirovna Bakina, Olga Vasiljeva, Georgy Mikhaylov, Boris Turk, Low-dimensional structures of organic and/or inorganic substances and use thereof, US10105318 (B2), US Patent and Trademark Office, 23. 10. 2018.

INTERNATIONAL PROJECTS

1. The Role of Cystatins in Neuroinflamamtion
   Asst. Prof. Nataša Kopitar – Jerala
   Slovenian Research Agency
2. Cancer management with cathepsin-targeting protein-drug conjugates: application to brain tumor therapies
   Prof. Boris Turk
   Slovenian Research Agency
3. COST BM1307; European Network to integrate Research on Intracellular Proteolysis Pathways in Health and Disease (PROTEOSTASIS)
   Prof. Boris Turk
   Cost Office
4. COST OC-2015; TRANSAUTOPHAGY: European Network of Multidisciplinary Research and Translation of Autophagy Knowledge
   Prof. Eva Žerovnik
   Cost Office
5. COST CA 15203; Mitochondrial Mapping: Evolution-Age-Gender-Lifestyle-Environment
   Asst. Prof. Nataša Kopitar – Jerala
   Cost Office
6. COST CA15124; NEUBIAS - A New Network of European Bioimage Analysts to Advance Life Science Imaging
   Asst. Prof. Tina Zavašnik Bergant
   Cost Office
7. COST CA15214; An Integrative Action for Multidisciplinary Studies on Cellular Structural Networks
   Asst. Prof. Nataša Kopitar – Jerala
   Cost Office

RESEARCH PROGRAMS

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

R & D GRANTS AND CONTRACTS

1. Structural insight into iodine metabolism
   Dr. Ajda Taler-verčič
2. Insights into the protein interactions involved in the Potato virus Y potatorelation
   Prof. Dušan Turk
3. Cathepsin X inhibitors impair the resistence of tumor cells to antiprotease therapy
   Prof. Boris Turk
4. Molecular genetic biomarkers and mechanisms of unresponsiveness to biological therapyanti-TNF in patients with chronic immune diseases
   Prof. Boris Turk
5. The role of micro RNA-21 and cathepsins in delayed preconditioning to acute kidney injury
   Prof. Boris Turk
6. Inhibition of Staphylococcus aureus cell wall remodeling
   Prof. Dušan Turk
7. Role of legumain in infection and inflammation
   Prof. Marko Fonović
8. Lyposomal Proteases in Semaphorin Signaling and Cell Polarity
   Prof. Boris Turk
   Icgeb - International Centre for Genetic
9. In Vivo Studies of the Potentiating Action of Micro-Mesoporous Powder Nanomaterials Containing Magnetic Iron Oxides
   Prof. Boris Turk
   Institute Of Strength Physics and Materials

VISITORS FROM ABROAD

1. Prof. dr. Jiři Neužil, Griffith University, Southport, Qld, Australia, 4-6 April 2018
2. Dr. Pal Tod, Semmelweis University, Hungary, 14 May 2018
3. Prof. dr. Sandra B. Gabelli, The Johns Hopkins University, Baltimore, Maryland, USA, 15-25 September 2018
4. Dr. Francesca Coscia, Cambridge Biomedical Campus, UK, 30 September to 3 October 2018
5. Prof. dr. Miquel Coll, IRB Barcelona, Spain, 28-30 October 2018

STAFF

Researchers

1. Dr. Iztok Dolenc
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3. Asst. Prof. Nataša Kopitar - Jerala
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20. Dr. Ajda Taler-Verčič
21. Dr. Aleksandra Usenik
22. Dr. Robert Vidmar
23. *Dr. Matej Vizovišek, left 01.11.18*
24. *Dr. Janja Završnik, left 01.11.18*

Postgraduates

25. Monika Biasizzo, B. Sc.
26. *Dr. Janja Božič, 01.10.18, transferred to Department B3*
27. Andreja Bratovš, B. Sc.
28. Marija Grozdanić, B. Sc.
29. Urban Javoršek, B. Sc.
30. *Aleksander Krajnc, B. Sc., left 01.05.18*
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32. *Dr. Sara Pintar, left 01.07.18*
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42. Polonca Pirš Kovačič
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BIBLIOGRAPHY

Original Article

1. Jure Borišek, Sara Pintar, Mitja Ogrizek, Dušan Turk, Andrej Perdih, Marjana Novič, "A water-assisted catalytic mechanism in glycoside hydrolases demonstrated on the Staphylococcus aureus autolysin E", *ACS catalysis*, 2018, **8**, 5, 4334-4345.
2. Marcin Poreba *et al.* (11 authors), "Selective imaging of cathepsin L in breast cancer by fluorescent activity-based probes", *Chemical science*, 2018, **9**, 8, 2113-2129.
3. Katerina Jazbec, Mojca Jež, Boštjan Smrekar, Simona Miceska, Jasmina Živa Rožman, Urban Švajger, Janja Završnik, Tadej Malovrh, Primož Rožman, "Chimerism and gene therapy - lessons learned from non-conditioned murine bone marrow transplantation models", *European journal of haematology*, 2018, **100**, 4, 372-382.
4. Jure Borišek, Sara Pintar, Mitja Ogrizek, Simona Golič Grdadolnik, Vesna Hodnik, Dušan Turk, Andrej Perdih, Marjana Novič, "Discovery of (phenylureido)piperidinyl benzamides as prospective inhibitors of bacterial autolysin E from Staphylococcus aureus", *Journal of enzyme inhibition and medicinal chemistry*, 2018, **33**, 1, 1239-1247.
5. Izraillevich Marat Lerner *et al.* (11 authors), "Crumpled aluminum hydroxide nanostructures as a microenvironment dysregulation agent for cancer treatment", *Nano letters*, 2018, **18**, 9, 5401-5410.
6. Samra Hasanbašić, Alma Jahić, Selma Berbić, Magda Tušek-Žnidarič, Eva Žerovnik, "Inhibition of protein aggregation by several antioxidants", *Oxidative medicine and cellular longevity*, 2018, 8613209.
7. Nikolai Engedall, Eva Žerovnik, Alexander Rudov, Francesco Galli, Fabiola Olivieri, Antonio Domenico Procopio, Maria Rita Rippo, Vladia Monsurrò, Michele Betti, Maria Cristina Albertini, "From oxidative stress damage to pathways, networks, and autophagy via microRNAs", *Oxidative medicine and cellular longevity*, 2018, 4968321.
8. Min Pan, Henrik Schinke, Elke Luxenburger, Gisela Kranz, Julius Shakhtour, Darko Libl, Yuanchi Huang, Aljaž Gaber, Miha Pavšič, Brigita Lenarčič, Julia Kitz, Mark Jakob, Sabina Schwenk-Zieger, Martin Canis, Julia Hess, Kristian Unger, Philipp Baumeister, Olivier Gires, "EpCAM ectodomain EpEX is a ligand of EGFR that counteracts EGF-mediated epithelial-mesenchymal transition through modulation of phospho-ERK1/2 in head and neck cancers", *PLoS biology*, 2018, **16**, 9, e2006624.
9. Janja Božič, Veronika Stoka, Iztok Dolenc, "Glucosamine prevents polarization of cytotoxic granules in NK-92 cells by disturbing FOXO1/ERK/paxillin phosphorylation", *PloS one*, 2018, **13**, 7, 0200757.
10. Aljaž Gaber, Seung Joong Kim, Robyn M. Kaake, Mojca Benčina, Nevan J. Krogan, Andrej Šali, Miha Pavšič, Brigita Lenarčič, "EpCAM homo-oligomerization is not the basis for its role in cell-cell adhesion", *Scientific reports*, 2018, **8**, 13269.

Review Article

1. Lorenzo Galluzzi *et al.* (168 authors), "Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018", *Cell death and differentiation*, 2018, **25**, 3, 486-541.
2. Matej Vizovišek, Robert Vidmar, Marcin Drag, Marko Fonović, Guy S. Salvesen, Boris Turk, "Protease specificity: towards in vivo imaging applications and biomarker discovery", *TiBS: Trends in biochemical sciences*, 2018, **43**, 10, 829-844.

Published Conference Contribution

1. Tjaša Tibaut, Tihomir Tomašič, Vesna Hodnik, Marko Anderluh, Sara Pintar, Marjana Novič, Dušan Turk, "Application of fragment based virtual screening towards inhibition of bacterial N-acetyglucosaminidase", In: Marjan Vračko (ed.), Marjana Novič (ed.), *18th International Conference on QSAR in Environmental and Health Sciences (QSAR 2018), June 11-15, Bled, Slovenia. Pt. 2*, (SAR and QSAR in environmental research **29**), 2018, 9, 647-660.

Independent Component Part or a Chapter in a Monograph

1. Livija Tušar, Marjana Novič, Marjan Tušar, Jure Zupan, "Structural elucidation", In: *Reference module in chemistry, molecular sciences and chemical engineering*, Jan Reedijk (ed.), [S. n.]: Elsevier, 2018.

Patent

1. Sergey Grigorievich Psakhie, Izraillevich Marat Lerner, Elena Alekseevna Glazkova, Olga Vladimirovna Bakina, Olga Vasiljeva, Georgy Mikhaylov, Boris Turk, *Low-dimensional structures of organic and/or inorganic substances and use thereof,* US10105318 (B2), US Patent and Trademark Office, 23. 10. 2018.
2. Norbert Schaschke, Olga Vasiljeva, Georgy Mikhaylov, Boris Turk, *Cathepsin-binding compound bound to a liposome and its diagnostic and therapeutic use,* EP2723387 (B1), European Patent Office, 18. 04. 2018.

Mentoring

1. Katja Bidovec, *Role of cathepsin D in TNFα- and menadione-induced apoptosis:* doctoral dissertation, Ljubljana, 2018 (mentor Veronika Stoka; co-mentor Vito Turk).
2. Janja Božič, *Role of glucosamine on the localization of cathepsins C and E and cytotoxicity of natural killer cells:* doctoral dissertation, Ljubljana, 2018 (mentor Veronika Stoka; co-mentor Iztok Dolenc).
3. Nežka Kavčič, *Different types of cell death and the role of reactive oxygen species in cell death signalling:* doctoral dissertation, Ljubljana, 2018 (mentor Boris Turk).
4. Sara Pintar, *Structural and biochemical characterization of autolysins from Staphylococcus aureus Mu50:* doctoral dissertation, Ljubljana, 2018 (mentor Dušan Turk).