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. A 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 an 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 signaling 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 signaling 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 Pharmacological Sciences. In this feature article we overviewed the progress and current trends in the area of cysteine cathepsins in disease management, including as drug targets, targets for noninvasive diagnostic imaging and for targeted drug delivery and as prodrug activators.

We have continued with proteomic approaches devoted to the identification of protease specificities and the identification of physiological protease substrates. We have thus developed a novel, first, gel-based, label-free, proteomic approach (DIPPS-direct in-gel profiling of protease specificity) that enables the quick and reliable determination of protease-cleavage specificities under a large variety of experimental conditions. The methodology is based on the in-gel digestion of the gel-separated proteome with the studied protease, the enrichment of cleaved peptides by gel extraction, and a subsequent mass-spectrometry analysis combined with a length-limited unspecific database search. We applied the methodology to profile ten proteases ranging from highly specific (trypsin, endoproteinase GluC, caspase-7, and legumain) to broadly specific (matrix-metalloproteinase-3, thermolysin, and cathepsins K, L, S, and V). Using DIPPS, we were able to perform specificity profiling of thermolysin at its optimal temperature of 75°C, which confirmed the applicability of the method for extreme experimental conditions. Additionally, DIPPS enabled the first global specificity profiling of legumain at an acidic pH, which revealed a pH-dependent change in the specificity of this protease, further supporting its broad applicability.

In addition to proteomic approaches, we also worked on small molecule substrates and 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 biotin-labelled inhibitors that targeted proteases. We developed a highly sensitive and adaptable donor/acceptor pair that can be used to investigate the substrate specificity of cysteine proteases, serine proteases and metalloproteinases. This novel pair comprises 7-amino-4-carbamoylmethylcoumarin (ACC) as the fluorophore and 2,4-dinitrophenyl-lysine (Lys(DNP)) as the quencher. Using caspase-3, caspase-7, caspase-8, neutrophil elastase, legumain, and two matrix metalloproteinases (MMP2 and MMP9), we demonstrated that substrates containing ACC/Lys(DNP) exhibit 7 to 10 times higher sensitivity than conventional 7-methoxycoumarin-4-yl acetic acid (MCA)/Lys(DNP) substrates; thus, substantially lower amounts of substrate and enzyme can be used for each assay. Therefore, the ACC/Lys(DNP) pair can be considered a novel and sensitive scaffold for designing the substrates for any group of endopeptidases. We further demonstrated that IQF substrates containing unnatural amino acids can be used to investigate protease activities/specificities for peptides containing post-translationally modified amino acids. Finally, we used IQF substrates to re-investigate the P1-Asp characteristic of caspases, thus demonstrating that some human caspases can also hydrolyze substrates after glutamic acid.

We also continued to work on targeted drug-delivery systems based on designed Ankyrin repeat proteins (DARPins). The development of highly selective and versatile small-molecule probes for cathepsins has been challenging; however, we were able to develop several cathepsin B-specific DARPins that have the potential for non-invasive diagnostic imaging and theranostic applications for cancer and inflammation.

The most potent was selective DARPin *8h6* , which inhibited cathepsin B in the picomolar range by binding to a site with low structural conservation in cathepsins, as revealed by the X-ray structure of the complex. DARPin *8h6* blocked cathepsin B activity in tumours *ex vivo* and was successfully applied in *in-vivo* optical imaging in two mouse breast-cancer models, in which cathepsin B was bound to the cell membrane or secreted to the extracellular milieu by tumour and stromal cells. Our approach validates cathepsin B as a promising diagnostic and theranostic target in cancer and other inflammation-associated diseases.

Besides drug-delivery systems for active targeting, we also worked on drug-delivery systems for oral application. As part of the FP7 Alexander project in collaboration with A. Azqueta (University of Navarra), we used an in-vivo imaging system, IVIS Spectrum, to validate the biodistribution of poly(anhydride) Gantrez® AN 119 (GN-MA-NP) nanoparticles in the gastro intestinal tract. We were able to determine the retention times for each segment of gastro intestinal tract and also confirmed the excretion of nanoparticles, therefore making GN-MA-NP a promising nanocarrier for oral drug-delivery systems.

Part of the work was also devoted to inhibitors. Since cysteine cathepsins, in addition to their important physiological functions, have been associated with multiple pathologies, including cancer. Therefore, we investigated their major and most potent inhibitor cystatin C that regulates the extracellular activity of cysteine cathepsins. We investigated the role of cystatin C in mammary cancer using CstC knockout mice and a mouse model of breast cancer induced by the expression of the polyoma middle T oncoprotein (PyMT) in the mammary epithelium. We showed that the ablation of CstC reduced the rate of mammary tumor growth. Notably, a decrease in the proliferation of CstC knockout PyMT tumor cells was demonstrated *ex vivo* and *in vitro*, indicating a role for this protease inhibitor in signaling pathways that control cell proliferation. An increase in phosphorylated p-38 was observed in CstC knockout tumors, suggesting a novel function for cystatin C in cancer development, independent of the TGF-β pathway. Moreover, a proteomic analysis of the CstC wild-type and knockout PyMT primary cell secretomes revealed a decrease in the levels of 14-3-3 proteins in the secretome of knock-out cells, suggesting a novel link between cysteine cathepsins, cystatin C and 14-3-3 proteins in tumorigenesis.

Additionally, some animals such as the tick, in order to ensure successful feeding, their saliva contains a number of inhibitory proteins, among which is also cystatin OmC2, that interfere with the host immune response and help to create a permissive environment for pathogen transmission. The potential targets of the salivary cystatins are two host cysteine proteases, cathepsin S, which is essential for antigen- and invariant chain-processing, and cathepsin C (dipeptidyl peptidase 1, DPP1), which plays a critical role in processing and activation of the granule serine proteases. To study salivary cystatin OmC2 from *Ornithodoros moubat*, we used differentiated MUTZ-3 cells as a model of immature dendritic cells of the host skin. Following internalization, cystatin OmC2 was initially found to inhibit the activity of several cysteine cathepsins, as indicated by the decreased rates of degradation of fluorogenic peptide substrates. To identify the targets, affinity chromatography was used to isolate His-tagged cystatin OmC2 together with the bound proteins from MUTZ-3 cells. Cathepsins S and C were identified in these complexes by mass spectrometry and confirmed by immunoblotting. We also observed a reduced increase in the surface expression of MHC II and CD86, which are associated with the maturation of dendritic cells. In contrast, human inhibitor cystatin C, which is normally expressed and secreted by dendritic cells, did not affect the expression of CD86.

Besides proteases we also investigated the conformational plasticity of myotilin, which is important in the organization and maintenance of Z-disk integrity. This involves direct binding to F-actin and filamin C, a function mediated by its Ig domain pair. While the structures of these two individual domains are known, information about their relative orientation and flexibility remains limited. We set out to characterize the Ig domain pair of myotilin with an emphasis on its molecular structure, dynamics and phylogeny.

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), 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 works resulting from these collaborations have already been published.

We were involved in the Slovenian Center of Excellence CIPKEBIP that we coordinate. In addition, there are numerous other international collaborations with excellent research teams from different countries, including Belgium (a joint project through FWO), Spain, France, Germany, Sweden, Switzerland, UK, USA, Australia, Hungary and Japan, which resulted in joint publications.

In addition, B. Turk organized an EMBO Workshop Mitochondria, Apoptosis, Cancer (MAC17) in Bled, and several members of the department were invited to give lectures at international symposia and foreign universities.

Some outstanding publications in the past year

1. Završnik J, Butinar M, Prebanda Trstenjak M, Krajnc A, Vidmar R, Fonovic M, Grubb A, Turk V, Turk B, Vasiljeva O (2017) Cystatin C deficiency suppresses tumor growth in a breast cancer model through decreased proliferation of tumor cells. Oncotarget 8, 73793-73809 doi: 10.18632/oncotarget.17379.
2. Zavašnik-Bergant T, Vidmar R, Sekirnik A, Fonovic M, Salát J, Grunclová L, Kopáček P, Turk B. (2017) Salivary Tick Cystatin OmC2 Targets Lysosomal Cathepsins S and C in Human Dendritic Cells. Front Cell Infect Microbiol. 7:288. doi: 10.3389/fcimb.2017.00288. eCollection 2017
3. Kramer L, Turk D, Turk B (2017) The future of cysteine cathepsins in disease management. Trends Pharmacol Sci, 38:873-898.
4. Kramer L, Renko M, Završnik J, Turk D, Seeger MA, Vasiljeva O, Grütter GG, Turk V, Turk B (2017) Non-invasive in vivo imaging of tumour-associated cathepsin B by a highly selective inhibitory DARPin. Theranostics, 7: 2806-2821.
5. Vidmar R, Vizovišek M, Turk D, Turk B, Fonović M (2017) Protease cleavage site fingerprinting by label-free in-gel degradomics reveals pH-dependent specificity switch of legumain. EMBO J. 36: 2455-2465.

Awards and Appointments

1. Vito Turk: Honorary Member of the Jožef Stefan Institute, Ljubljana, Slovenia, 26. 10. 2017
2. Eva Vidak: Prešeren Award at the University of Ljubljana, Ljubljana, Slovenia, 6 December 2017, Preparation of recombinant human caspase-1 and identification of its extracellular substrates

Organisation of conferences, congresses and meetings

1. 34th Winter School on Proteinases and Inhibitors 2017, Tiers, Italy, 8–12 March 2017, co-organisers
2. Mitochondria, Apoptosis and Cancer (MAC 2017), Bled, Slovenia, 15–18 September 2017

Patents granted

1. Henry Berbard Lowman, Luc R. Desnoyers, Shouchun Liu, James William West, Jason Sagert, Olga Vasiljeva, Elizabeth Menendez, Activatable antibodies that bind epidermal growth factor receptor and methods of use thereof, US9545442 (B2), US Patent Office, 17. 01. 2017.
2. Olga Vasiljeva, Georgy Mikhaylov, Boris Turk, Norbert Schaschke, Cathepsin-binding compounds bound to a carrier and their diagnostic use, US9827337 (B2), US Patent Office, 28. 11. 2017.

INTERNATIONAL PROJECTS

1. COST BM1307; European Network to integrate Research on Intracellular Proteolysis Pathways in Health and Disease (PROTEOSTASIS)
   Prof. Boris Turk
   Cost Office
2. COST OC-2015; TRANSAUTOPHAGY: European Network of Multidisciplinary Research and Translation of Autophagy Knowledge
   Prof. Eva Žerovnik
   Cost Office
3. COST CA 15203; Mitochondrial Mapping: Evolution-Age-Gender-Lifestyle-Environment
   Asst. Prof. Nataša Kopitar – Jerala
   Cost Office
4. COST CA15124; NEUBIAS - A New Network of European Bioimage Analysts to Advance Life Science Imaging
   Asst. Prof. Tina Zavašnik Bergant
   Cost Office
5. COST CA15214; An Integrative Action for Multidisciplinary Studies on Cellular Structural Networks
   Asst. Prof. Nataša Kopitar – Jerala
   Cost Office
6. Building Interface between Crystallographic Software MAIN and Integrative Modeling Platform IMP
   Prof. Dušan Turk
   Slovenian Research Agency
7. Effect of Anti-oxidants on Protein Aggregation; In Vitro Study of Amyloid Fibrillation on the Model of Stefin B and Beta2-microglobulin
   Prof. Eva Žerovnik
   Slovenian Research Agency
8. Cancer management with cathepsin-targeting protein-drug conjugates: application to brain tumor therapies
   Prof. Boris Turk
   Slovenian Research Agency

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. Proteases in inflammation and cell death
   Prof. Boris Turk
5. Role of cysteine cathepsins in inflammation-associated diseases
   Prof. Boris Turk
6. The role of micro RNA-21 and cathepsins in delayed preconditioning to acute kidney injury
   Prof. Boris Turk

7. Inhibition of Staphylococcus aureus cell wall remodeling
   Prof. Dušan Turk
8. Enabling technology for high-quality piezoMEMS
   Prof. Boris Turk
   Ministry of Education, Science and Sport
9. Lyposomal Proteases in Semaphorin Signaling and Cell Polarity
   Prof. Boris Turk
   Icgeb - International Centre For Genetic
10. Proteomic analysis
    Prof. Marko Fonović

NEW CONTRACT

1. Mass spectromety analysis
   Prof. Boris Turk
   Krka, Tovarna Zdravil, d. d.

VISITORS FROM ABROAD

1. Samra Hasanbašić, Univerzitet Tuzla,Tuzla, Bosnia and Herzegovina, 18. 9.– 17. 11. 2017
2. Alma Jahić, Univerzitet Tuzla, Tuzla, Bosnia and Herzegovina, 20. 3.– 28. 5. 2017

STAFF

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6. Andrej Šali, B. Sc.
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13. Dr. Miha Butinar
14. *Dr. Maruša Hafner Česen, left 01.07.17*
15. Dr. Katarina Karničar
16. Dr. Lovro Kramer
17. Dr. Nataša Lindič
18. Dr. Jure Pražnikar*
19. Dr. Vida Puizdar
20. *Dr. Jelena Rajković, left 06.07.17*
21. *Dr. Barbara Sobotič, left 13.03.17*
22. Dr. Ajda Taler-Verčič
23. Dr. Aleksandra Usenik
24. Dr. Robert Vidmar
25. Dr. Matej Vizovišek
26. Dr. Janja Završnik

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28. Janja Božič, B. Sc.
29. Andreja Bratovš, B. Sc.
30. Marija Grozdanić, B. Sc.
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32. Urban Javoršek, B. Sc.
33. Aleksander Krajnc, B. Sc.
34. Jure Loboda, B. Sc.
35. Sara Pintar, B. Sc.
36. Mojca Trstenjak Prebanda, B. Sc.
37. Eva Vidak, B. Sc.

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38. *Marjeta Arnolj, B. Sc., left 01.08.17*
39. Nežka Kavčič, B. Sc.
40. Andreja Sekirnik, B. Sc.
41. Ivica Štefe, B. Sc.

Technical and administrative staff
42. Maja Orehek, B. Sc.
43. Dejan Pelko
44. Polonca Pirš Kovačič
45. *Barbara Vrtačnik, left 31.12.17*

Note:
* part-time JSI member

BIBLIOGRAPHY

Original Article

1. Yael Ben-Nun, Gait Fichman, Lihi Adler-Abramovich, Boris Turk, Ehud Gazit, Galia Blum, "Cathepsin nanofiber substrates as potential agents for targeted drug delivery", *J. control. release*, **257**, 60-67, 2017.
2. Katja Bidovec, Janja Božič, Iztok Dolenc, Boris Turk, Vito Turk, Veronika Stoka, "Tumor necrosis factor-α induced apoptosis in U937 cells promotes cathepsin D-independent stefin B degradation", *J Cell Biochem*, **118**, 12, 4813-4820, 2017.
3. Vashendriya V. V. Hira, Urška Verbovšek, Barbara Breznik, Matic Srdič, Marko Novinec, Hala Kakar, Jill Wormer, Britt van der Swaan, Brigita Lenarčič, Luiz Juliano, Shwetal Mehta, Cornelis J. F. van Noorden, Tamara Lah Turnšek, "Cathepsin K cleavage of SDF-1[alpha] inhibits its chemotactic activity towards glioblastoma stem-like cells", *Biochim. biophys. acta, Mol. cell res.*, **1864**, 3, 594-603, 2017.
4. T. Iglesias, J. M. Irache, Miha Butinar, Boris Turk, A. López de Cerain, A. Azqueta, "Genotoxic evaluation of poly(anhydride) nanoparticles in the gastrointestinal tract of mice", *Int. j. pharm.*, issues. 1-2, **530**, 187-194, 2017.
5. Lovro Kramer, Miha Renko, Janja Završnik, Dušan Turk, Markus A. Seeger, Olga Vasiljeva, Markus G. Grütter, Vito Turk, Boris Turk, "Non-invasive in vivo imaging of tumour-associated cathepsin B by a highly selective inhibitory DARPin", *Theranostics*, **7**, 11, 2806-2821, 2017.
6. Marko Mihelič, Kristina Vlahoviček-Kahlina, Miha Renko, Stephan Mesnage, Andreja Doberšek, Ajda Taler-Verčič, Andreja Jakas, Dušan Turk, "The mechanism behind the selection of two different cleavage sites in NAG-NAM polymers", *IUCrJ*, **4**, part 2, 185-198, 2017.
7. Marcin Poreba *et al.* (13 authors), "Highly sensitive and adaptable fluorescence-quenched pair discloses the substrate specificity profiles in diverse protease families", *Sci. rep.*, **7**, 43135, 2017.
8. Jure Pražnikar, "Particulate matter time-series and Köppen-Geiger climate classes in North America and Europe", *Atmos. environ.*, **150**, 136-145, 2017.
9. Vid Puž, Miha Pavšič, Brigita Lenarčič, Kristina Djinović Carugo, "Conformational plasticity and evolutionary analysis of the myotilin tandem Ig domains", *Sci. rep.*, **7**, 3993, 2017.
10. Clifford Taggart *et al.* (12 authors), "Protean proteases: at the cutting edge of lung diseases", *Eur Respir J*, **49**, 2, 1501200, 2107.
11. Ajda Taler-Verčič, Samra Hasanbašić, Selma Berbić, Veronika Stoka, Dušan Turk, Eva Žerovnik, "Proline residues as switches in conformational changes leading to amyloid fibril formation", *Int. j. mol. sci.*, **18**, 3, 549, 2017.
12. Aleksandra Usenik, Miha Renko, Marko Mihelič, Nataša Lindič, Jure Borišek, Andrej Perdih, Gregor Pretnar, Uwe Müller, Dušan Turk, "The CWB2 cell wall-anchoring module is revealed by the crystal structures

of the Clostridium difficile cell wall proteins Cwp8 and Cwp6", *Structure (Lond.)*, **25**, 3, 514-521, 7 Mar. 2017.
13. Robert Vidmar, Matej Vizovišek, Dušan Turk, Boris Turk, Marko Fonović, "Protease cleavage site fingerprinting by label-free in-gel degradomics reveals pH-dependent specificity switch of legumain", *EMBO j.*, **36**, 16, 2455-2465, 2017.
14. Danijela Vujošević, Uroš Cvelbar, Urška Repnik, Martina Modic, Saša Lazović, Tina Zavašnik-Bergant, Nevena Puač, Boban Mugoša, Evangelos Gogolides, Zoran Lj. Petrović, Miran Mozetič, "Plasma effects on the bacteria Escherichia coli via two evaluation methods", *Plasma Sci. Tech.*, **19**, 7, 075504, 2017.
15. Tina Zavašnik-Bergant, Robert Vidmar, Andreja Sekirnik, Marko Fonović, Jiří Salát, Lenka Grunclová, Petr Kopáček, Boris Turk, "Salivary tick cystatin OmC2 targets lysosomal cathepsins S and C in human dendritic cells", *Front. cell. infect. microbiol.*, **7**, 288, 2017.
16. Janja Završnik, Miha Butinar, Mojca Trstenjak-Prebanda, Aleksander Krajnc, Robert Vidmar, Marko Fonović, Anders Grubb, Vito Turk, Boris Turk, Olga Vasiljeva, "Cystatin C deficiency suppresses tumor growth in a breast cancer model through decreased proliferation of tumor cells", *Oncotarget*, **8**, 73793-73809, 2017.
17. Eva Žerovnik, "Co-chaperoning by amyloid-forming proteins: cystatins: cystatins vs. crystallins", *Eur. biophys. j.*, **46**, 8, 789-793, 2017.
18. Eva Žerovnik, "Putative alternative functions of human stefin B (cystatin B): binding to amyloid-beta, membranes, and copper", *JMR, J. mol. recognit.*, **30**, 1, e2562, 2017.

Review Article

1. Nežka Kavčič, Katarina Pegan, Boris Turk, "Lysosomes in programmed cell death pathways: from initiators to amplifiers", *Biol Chem*, **398**, 3, 289-301, 2017.
2. Nataša Kopitar-Jerala, "The role of interferons in inflammation and inflammasome activation", *Front. immunol.*, **8**, 1-9, 2017.
3. Lovro Kramer, Dušan Turk, Boris Turk, "The future of cysteine cathepsins in disease management", *Trends pharmacol. sci. (Regul. ed.)*, **38**, 10, 873-898, 2017.

Independent Component Part or a Chapter in a Monograph

1. Dušan Turk, "Boxes of model building and visualization", In: *Protein crystallography: methods and protocols*, (Methods in molecular biology, **1607**), (Springer protocols), Alexander Wlodawer, ed., Zbigniew Dauter, ed., Mariusz Jaskolski, ed., 2017, 491-548.
2. Matej Vizovišek, Robert Vidmar, Marko Fonović, "FPPS: Fast Profiling of Protease Specificity", In: *Protein terminal profiling: methods and protocols*, (Methods in molecular biology, **1574**), Oliver Schilling, ed., 2017, 183-196.
3. Yinliang Yang, Marko Fonović, Steven H. L. Verhelst, "Cleavable linkers in chemical proteomics application", In: *Activity-based proteomics: methods and protocols*, (Methods in molecular biology, volume **1491**), Herman S. Overkleeft, ed., Bogdan I. Florea, ed., 2017, 185-203.

Patent

1. Henry Berbard Lowman, Luc R. Desnoyers, Shouchun Liu, James William West, Jason Sagert, Olga Vasiljeva, Elizabeth Menendez, *Activatable antibodies that bind epidermal growth factor receptor and methods of use thereof,* US9545442 (B2), US Patent Office, 17. 01. 2017.
2. Olga Vasiljeva, Georgy Mikhaylov, Boris Turk, Norbert Schaschke, *Cathepsin-binding compounds bound to a carrier and their diagnostic use,* US9827337 (B2), US Patent Office, 28. 11. 2017.

Mentoring

1. Lovro Kramer, *Targeting cysteine cathepsins in inflammatory diseases:* doctoral dissertation, Ljubljana, 2017 (mentor Boris Turk).
2. Janja Završnik, *The role of stefin B and cystatin C knockout on cancer progression and metastasis in mouse model:* doctoral dissertation, Ljubljana, 2017 (mentor Vito Turk; co-mentor Olga Vasiljeva).