DEPARTMENT OF MOLECULAR AND BIOMEDICAL SCIENCES

The research program of the Department of Molecular and Biomedical Sciences is focused mainly on basic research in protein biochemistry, molecular and cellular biology, and genetics. The primary goal of our investigations is the acquisition of new understanding of mammalian pathophysiology with the aim of improving human and animal health.

Toxinology

With intensity, we have been studying the nose-horned viper (*Vipera a. ammodytes*) venom proteins that affect the blood coagulation process—haemostasis, in particular two such proteins, a serine protease homologue with anticoagulant activity (Vaa-SPH-1) and a serine protease with procoagulant, FVIIa-like activity (SP-10).

Components of the intrinsic blood coagulation pathway, among them FVIIIa, have been recognized as suitable therapeutic targets to treat venous thromboembolism, the pathological process behind two very serious cardiovascular diseases, deep vein thrombosis and pulmonary embolism. In collaboration with researchers from the National University of Singapore, the University Medical Center Ljubljana, the North Carolina Agricultural and Technical State University, and Novartis, Basel, Switzerland, we succeeded to conclude and to publish the work, in which we described a unique glycoprotein from the nose-horned viper venom, Vaa-SPH-1. This molecule, which is structurally an enzymatically inactive serine protease homologue, exhibits a potent anticoagulant action in human blood (Z. Latinović et al., *Thromb. Haemost.*, 118 (2018), 1713–1728). We demonstrated that one of its targets in the blood coagulation system is factor VIIIa (FVIIIa) of the intrinsic tenase complex, where it antagonizes the binding of FIXa. Anticoagulants with such characteristics are intensively sought, as they would be much safer for medical application than the current drugs, which frequently induce excessive bleeding and other complications. Vaa-SPH-1 represents a very promising template to design low-molecular-mass FVIIIa-directed anticoagulant substances, based on structural features of the interaction surface between Vaa-SPH-1 and FVIIIa. To this end, we constructed a three-dimensional model of Vaa-SPH-1 bound to FVIIIa and proposed the most appropriate structural elements of Vaa-SPH-1 to constitute small FVIIIa-binding molecules, potential new generation of anticoagulants (Figure 1).

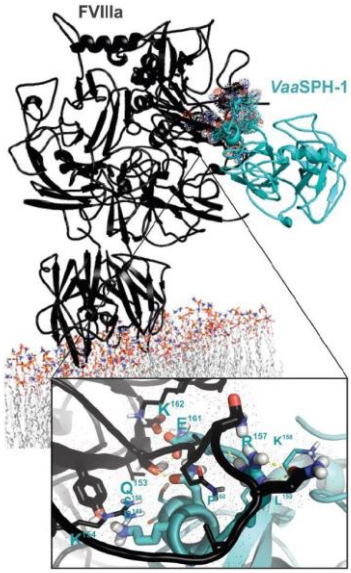

Figure 1. **The intrinsic tenase complex with Vaa-SPH in place of factor IXa.** The human intrinsic tenase consists of FIXa and FVIIIa (grey) complexed on the negatively charged plasma membrane of a platelet. In the intrinsic tenase complex, the atomic coordinates of FIXa were replaced with the coordinates of Vaa-SPH (blue) and docked by High Ambiguity Driven protein–protein DOCKing (HADDOCK) to generate thermodynamically most optimal structure. The contact area between FVIIIa and Vaa-SPH is zoomed in. Amino acid residues involved in major electrostatic interactions are indicated by large letters while those contributing less to the interaction by smaller. The figure is adapted from Z. Latinović et al. (*Thromb. Haemost.*, 118 (2018), 1713–1728).

The importance of this work is indicated by its immediate recognition, first in the professional community (e.g., by the journals Gene Therapy Weekly, Atlanta, USA and Medicina

danes, Ljubljana, Slovenia), and then also by the general public media. To the latter, the project leader, Professor I. Križaj, has been interviewed by the national Radio (Val 202 – Ime tedna (Figure 2); Radio Slovenia 1 – Aktualno), the national TV (TV Slovenia 1 – Dnevnik and Odmevi) and the commercial TV (POP TV – 24 ur).

Figure 2. **Description of an original anticoagulant molecule attracted also the general public.** The international research consortium led by Professor I. Križaj discovered and described an anticoagulant protein in the venom of the nose-horned viper with a potent anticoagulant activity in human blood. Due to its unique structure and mode of action, it has a great potential for the development of an innovative drug to treat venous thromboembolism. The discovery echoed in the professional as well as in the general public audience. The latter is reflected also in selection of the project leader as a name of the week on the national radio Val 202.

The work on Vaa-SP-10, the procoagulant venom serine protease with the FVIIa-like activity, substantially advanced in 2018 and we started with the preparation of a publication.

One of our traditional research topics in the field of toxinology is the study of molecular mechanisms of toxic action of secreted phospholipases A_2 ($sPLA_2$) from animal venoms. In particular, we are focused on those endowed with presynaptic neurotoxicity (β-neurotoxins). The knowledge that we are gaining by studying toxic $sPLA_2$s is helping us to discover the pathophysiological roles of orthologous mammalian $sPLA_2$s, for example, their role in the development of neurodegenerative diseases such as Alzheimer's disease.

Ammodytoxin (Atx) is a neurotoxic $sPLA_2$ from the venom of the nose-horned viper. The result of its action on the motor neuron is the inhibition of secretion of the neurotransmitter acetylcholine into the synaptic cleft and the flaccid paralysis of skeletal muscle. Among specific effects of Atx on nerve cells are damaged mitochondria and the damage inflicted is very similar to that induced by structurally homologous endogenous group IIA $sPLA_2$ when its activity is elevated, as, for example, in the early phase of Alzheimer's disease. Using Atx, we have detected the $sPLA_2$ receptor R25 in neuronal mitochondria. We developed the protocol for its purification and identified it as the subunit II of cytochrome c oxidase (CCOX), an essential constituent of the respiratory chain complex. We confirmed CCOX as the first intracellular membrane receptor for $sPLA_2$ by alternative Atx-affinity-labelling of purified CCOX and by demonstrating the encounter of Atx and CCOX in PC12 cells. This discovery suggests the explanation of the mechanism by which β-neurotoxins hinder production of ATP in poisoned nerve endings. It also provides a new insight into the potential function and dysfunction of an endogenous group IIA $sPLA_2$ in mitochondria. In 2018, the manuscript describing the first intracellular membrane receptor of an $sPLA_2$ molecule was prepared and accepted for publication in the esteemed journal Scientific Reports (J. Šribar et al., *Sci. Rep.*, (2018), in press).

In the area of $sPLA_2$ research, we also succeeded to receive a new international project (BI-RU/19-20-029). With our Russian partners from the Laboratory of Molecular Toxinology at Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry Russian Academy of Sciences, Moscow, for the next two years we will be studying, how endogenous $sPLA_2$s modulate functions of nicotinic acetylcholine receptor.

In 2018, we systematically analysed two snake venoms (venomics), the one from the nose-horned viper and the other from the very rare Croatian karst viper (*Vipera ursinii*).

To aid improving the current antivenom therapy towards higher specificity and efficiency, and to facilitate drug discovery, we constructed, by combining transcriptomic and proteomic analyses, the most comprehensive library of the nose-horned viper venom proteins and peptides. Of particular interest, a transcript coding for a protein similar to P-III SVMPs but lacking the catalytic, MP domain was also found at the protein level in the venom. We started to prepare the manuscript, which will be submitted for publication in 2019.

We determined the proteomic profile of the Croatian karst viper venom and, together with our colleagues from the University of Zagreb also its biological activities. This venom is much less toxic than that from the nose-horned viper and also the pattern of mice dying due to this venom is different. Experiments suggest the presence of a strong neurotoxic component in the Croatian karst viper venom, however, our studies excluded the presence of basic $sPLA_2$s, the only known neurotoxic components in the genus *Vipera* venoms. This suggests a discovery of a novel type of neurotoxic molecule in *Vipera* venoms.

In 2018, we reported together with the colleagues from the University Hospital of Split and the University of Split, Croatia, two unique cases of poisoning with the nose-horned viper venom in which, for the first time, thrombocytopenic purpura in patients has been detected (B. Lukšić et al., *Medicine*, 97 (2018), e13737). These unexpected clinical findings were characterized by unusually profound thrombocytopenia of the patients and purpura, observed on the face and thorax of both individuals. In most serious cases, such pathology can be even life threatening if not promptly recognized and treated. This is an important message to clinicians to consider possibility of such complication also in the case of nose-horned viper envenomation.

In collaboration with the colleagues from the Centre for Clinical Toxicology and Pharmacology, University Medical Centre Ljubljana, we investigated an interesting clinical effect, a profound and transient thrombocytopenia of functional platelets in patients envenomed by the nose-horned viper venom. In thromboembolic diseases, such as myocardial infarction and ischemic stroke, platelets play a pivotal role. Currently used antiplatelet drugs have one common side effect—a decreased count of platelets with inhibited function. Such condition represents a high risk of life-threatening haemorrhage especially in interventional cardiology and angiology employing antithrombotic approach. Our observation may pave the way to the development of a new group of antiplatelet agents, which will minimize the risk of life-threatening bleeding in antithrombotic approach in interventional cardiology and angiology, and increase the effectiveness of vessel dilatation and emboli aspiration. To this end, we demonstrated in 2018 that reversible thrombocytopenia in patients after poisoning with the nose-horned viper venom is caused by snake C-type lectin-like proteins (snaclecs). From the venom, we isolated a pool of these proteins and, by using flow cytometry, revealed that such effect is the consequence of their specific interaction with the platelet GPIb receptor. We also showed that GPIIb/IIa and P-selectin did not express on the membrane of these cells, confirming that the platelets did not undergo the activation.

Last year was also the first one of performing the Slovenian-Serbian bilateral project BI-RS/18-19-005 (“Characterization of new bacterial enzymes to ameliorate food quality and human health“). Partners exchanged visits in the collaborating institutions. With colleagues from the Institute of Molecular Genetics and Genetic Engineering, Belgrade, we pursued research activities in three main directions as follows. (1) To determine the specificity of cleavage of soybean trypsin inhibitor by the bacterial protease, (2) to analyse high molecular mass bacterial proteases and (3) to establish the most appropriate expression system for preparation of a recombinant snake venom CRISP.

At the end of 2018, we initiated a large research project with the collaboration of two foreign groups, the Department of Biotechnology and Biomedicine from the Technical University of Denmark and the Beijing Genomics Institute from Hong Kong. The major aim of the project is to

sequence, assemble *de novo*, annotate and thoroughly analyse the complete *Vipera a. ammodytes* genome.

As experts from the field of toxinology, we have been invited as lecturers on expert meetings and scientific conferences. Most worth mentioning are the invitations to D. Kordiš and I. Križaj to deliver keynote lectures at the 19th Congress of the European Section of the International Society on Toxinology, Yerevan, Armenia. We were also invited by the Slovenian journal Medicinski razgledi to prepare a review paper on venomous snakes in Slovenia (V. Leban et al., *Med. razgl.*, (2018), in press).

Lipid metabolism and signalling

Our work in this field is focused on identifying the cellular pathways of lipid acquisition and utilization that may be targeted to reduce the resistance of cancer cells to stress. The survival of cancer cells during severe stress depends on the availability of extracellular lipids and on their capacity to synthesize, mobilise or recycle their own intracellular lipids. By studying the ways in which cancer cells use lipids, we aim to reduce their remarkable ability to adapt to the inhospitable tumour microenvironment and thus reduce tumour growth, metastasis and resistance to therapy.

In our recent study (E. Jarc et al., *Biochim. Biophys. Acta – Mol. Cell Biol. Lipids*, 1863 (2018), 247–265) we report of a novel mechanism of lipid droplet-mediated protection of cancer cells against metabolic stress. The study provides evidence for a pro-survival and antioxidant role of lipid droplets that orchestrate unsaturated fatty acid storage and trafficking according to cellular needs (Figure 3).

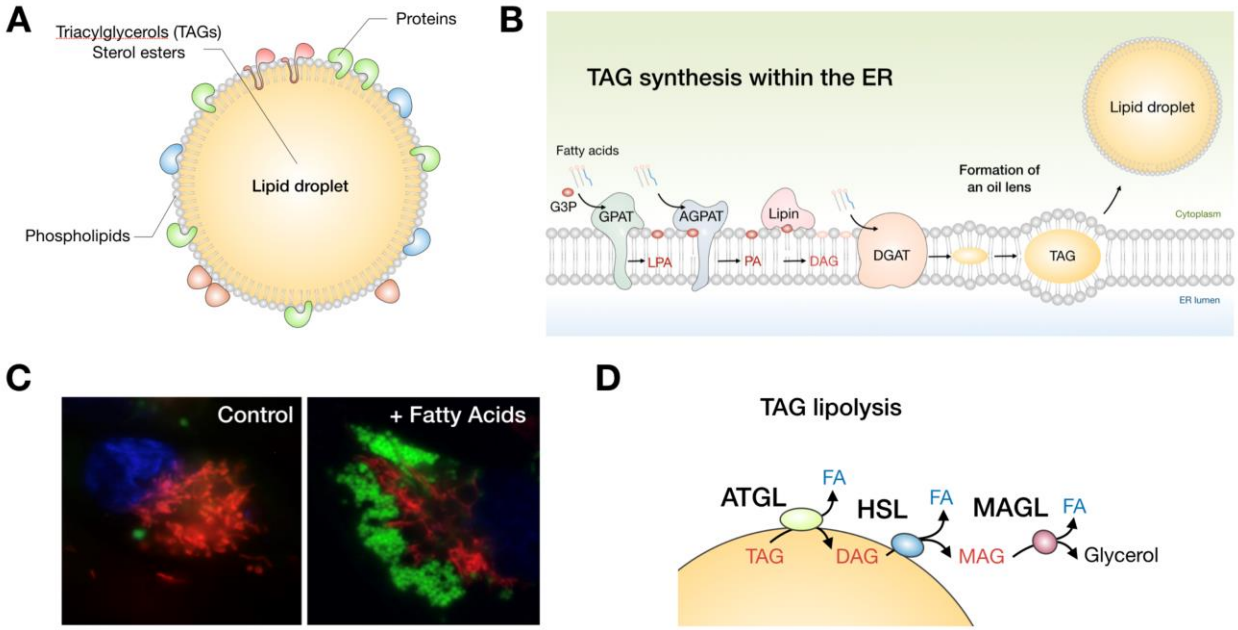

Figure 3. **Lipid droplet basics.** (A) Lipid droplets are composed of a central hydrophobic core of neutral lipids, mostly triacylglycerols (TAGs) and sterol esters, surrounded by a monolayer of phospholipid molecules, wherein numerous proteins with various functions are embedded. (B) TAG synthesis occurs in the endoplasmic reticulum membrane by sequential addition of fatty acids (FAs) (in their activated acyl-CoA form) to a glycerol-3-phosphate backbone, yielding lysophosphatidic acid (LPA), phosphatidic acid (PA) and diacylglycerol (DAG). These reactions are catalysed by several acyltransferase enzymes, including glycerol-3-phosphate acyltransferases (GPATs), acylglycerol-3-phosphate acyltransferases (AGPATs), and

phosphatidic acid phosphatases (lipins). This pathway is also responsible for the synthesis of phospholipids in the cell. The last and committed step in the TAG synthesis cascade is catalysed by the diacyglycerol acyltransferase enzymes (DGATs). (C) Lipid droplet biogenesis is induced in most cells exposed to exogenous FAs. This example shows breast cancer cells exposed to oleic acid. Lipid droplets (green) were stained with BODIPY 493/503, mitochondria (red) with Mitotracker Red and nuclei (blue) with DAPI and imaged with epifluorescence microscopy. (D) TAG lipolysis occurs by the sequential action of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) and leads to lipid droplet shrinkage and breakdown.

We describe a central role of triglycerides stored in lipid droplets in enabling protection from nutrient limitation, oxidative stress and fatty acid lipotoxicity. By silencing the rate-limiting enzyme in lipolytic lipid droplet breakdown, adipose triglyceride lipase (ATGL), by inhibiting lipid droplet biogenesis and by modulating the unsaturation levels of triglycerides stored in lipid droplets, we show that lipid droplets protect sensitive ω-3 and ω-6 polyunsaturated fatty acids from oxidation by storing them in the form of inert triglycerides, while concurrently providing fatty acids for mitochondrial energy production, redox homeostasis and cell survival. Our lipidomic analyses performed in collaboration with the group of Dr. Robert Zimmermann from the University of Graz, Austria, revealed that fatty acid lipotoxicity may be modulated in aggressive cancer cells by two complementary mechanisms: (1) PLA_2-induced lipid droplet biogenesis that finely tunes the fatty acyl composition of triglycerides stored within and (2) inhibition of ATGL-mediated lipolysis that leads to retention of fatty acids within lipid droplets. The lipidomic data was additionally published in full in a separate publication (E. Jarc et al., *Data Brief*, 18 (2018), 234–240). These findings are important for the emerging field of lipid metabolism in cancer, but they also have wider implications for lipid droplet biology and the regulation of nutrient stress in general, which is relevant in various pathophysiological contexts and diseases.

In our comprehensive review with the title "Lipid Droplets in Cancer: Guardians of Fat in a Stressful World" (T. Petan et al., *Molecules*, 23 (2018), e1941), we focused on recent advances describing the involvement of lipid droplets in the protection against nutrient, lipotoxic and oxidative stress in cancer cells and beyond. We discussed the emerging mechanisms of stress-induced lipid droplet biogenesis, the roles of lipid droplets during stress and the complex bidirectional relationship between lipid droplets and autophagy. Because studies on the role of lipid droplets in cancer are relatively scarce, we have provided a broader overview of lipid droplet function in cellular stress in various tissues and pathophysiological settings. Our discussion integrates many of these recently discovered principles of lipid droplet biology that can improve our understanding of the mechanisms that govern cancer cell adaptability and resilience to stress.

Our work in the field of lipid droplets was well accepted in the scientific community as judged by several invited lectures, most notably at the 14th GERLI Lipidomics Meeting – GERLI 40th Anniversary on the topic of "Biogenesis and Fate of Lipid Droplets", held in St. Maximin la Saint-Baume, France, where Dr. Petan was invited to present a keynote lecture. Our contributions were also selected for oral presentations at several internationally renowned meetings, including the conference on "Bioactive Lipids – from Chemistry to Biology and Medicine", held in Athens, Greece (T. Petan) and at the 7th European Workshop on Lipid Mediators (7EWLM), held in Brussels, Belgium (E. Jarc). Our PhD student Eva Jarc also received a FEBS YTF Travel Grant to attend the FEBS Advance Course "Lipid Dynamics and Membrane Contact Sites", held on the island of Spetses, Greece. Our work was also presented locally at a more public oriented meeting within the 2nd Physiology Day organized by the Slovenian Physiological Society on the occasion of the Nobel Prize for Physiology or Medicine award ceremony and held at the Slovenian Academy of Arts and Sciences, Ljubljana, where Dr. Petan was invited to give a talk on the importance of lipid droplets in cancer.

High-throughput genetics and functional genomics in yeast *Saccharomyces cerevisiae*; genomics, molecular biology and physiology of extremophilic and extremotolerant yeasts

Polygenic trait analysis and genome editing methods are among the fastest developing fields in genetics. In a multi-year project on the yeast lipid content analysis, a biotechnologically important polygenic trait, we found three new causative genes for this trait: *PIG1*, *PHO23* and *RML2* (M. Ogrizović et al., unpublished). We also developed improvements to the CRISPR-Cas9 method for the yeast genome editing (G. Žun and U. Petrovič, unpublished). In collaboration with the research group of Dr. Gohil from the Texas A & M University, USA, we showed that ethanolamine allows partial activity of mitochondria in yeast cells that do not contain cardiolipin (W. Basu Ball et al., *J. Biol. Chem.*, 293 (2018), 10870–10883). This achievement was possible because of our previously developed method for determining the chemogenomic interactions of yeast genes.

Studies of mechanisms enabling survival at extreme values of physico-chemical parameters are often performed on model microorganisms, but they can be complemented by studies of extremophilic and extremotolerant yeasts. In addition to improving our understanding of the ecology of extreme environments such research importantly expands the possibilities for exploitation of the substantial biotechnological potentials of microorganisms from such environments. Since these are mostly non-model species and *in vitro* work with them is often non-trivial, we focused primarily on genomic analyses in order to establish a resource for future research. Based on comparative genomics of extremophilic and extremotolerant yeasts we confirmed the correlation between the ability to grow at high salt concentrations and resistance to oxidative stress in halotolerant species, while the halophilic *Wallemia ichthyophaga* (with a different stress tolerance strategy) did not conform to this observation (C. Gostinčar and N. Gunde-Cimerman, *Genes*, 9 (2018), 143). We studied the glycerol metabolism of two polyextremotolerant yeast species, *Aureobasidium pullulans* and *Aureobasidium subglaciale* (Figure 4) (M. Turk and C. Gostinčar, *Fungal Biol.*, 122 (2018), 63–73).

Figure 4. **Polyextremotolerant black yeasts of the genus *Aureobasidium*.**

By comparing the genomes of the extremely halotolerant *Hortaea werneckii* we found strong indications of hybridisation within the species (C. Gostinčar et al., *BMC Genomics*, 19 (2018), 364). We used comparative genomics to study the link between opportunistic pathogenicity and poliextremotolerance in 20 species of black yeasts and confirmed the observations with a large-scale phylogenetic analysis (C. Gostinčar et al., *Fungal Fungal Divers.*, 93 (2018), 195–213). In collaboration with the group of Dr. Daly from the Uniformed Services University of the Health Sciences, Bethesda, USA, we studied the bioremediation potential of the acidotolerant and radiotolerant yeast *Rhodotorula taiwanensis* (R. Tkavc et al., *Frontiers Microbiol.*, 8 (2018), 2528). In collaboration with the group of Dr. Rodrigues from São Paulo State University, Brazil,

we analysed the thermotolerance of proteases of thermotolerant and thermophilic fungi on a genetic level (T.B. de Oliveira et al., *BMC Genomics*, 19 (2018), 152).

Evolutionary genomics

Yarrowia lipolytica is an oleaginous yeast that can store more than 20% of triacylglycerol (TAG) in its biomass, therefore it is an attractive host for the production of single cell oil. Oleaginous microorganisms store high amounts of TAG in intracellular lipid droplets (LDs). In this work, we characterized a protein of the oleaginous yeast *Y. lipolytica* that is associated with LD and plays a role in the regulation of TAG storage (G. Bhutada et al., *Biochim. Biophys. Acta – Mol. Cell Biol. Lipids*, 1863 (2018), 1193–1205). This protein is required for the oleaginous phenotype of *Y. lipolytica* because deletion of the coding gene results in a strongly reduced TAG content of the mutant. Therefore, we named it oleaginicity-inducing LD protein, Oil1. Phylogenomic and phylogenetic analysis demonstrated that Oil1 is a member of the Sps4 family of proteins, which is restricted to the true yeasts (Saccharomycotina). The analysis of secondary structures in fungal LD-associated proteins (Oil1, Sps4, Pet10 and Mpl1) has shown that they consist exclusively of alpha helices. The numbers and the sizes of the helices among the fungal LD-associated proteins vary considerably, and they share no sequence similarity. The analysis of the helices in the Oil1 protein has shown the presence of four amphipathic helices. The 3D structure model of Oil1 protein shows an extended structure (Figure 5), suggesting bendability of the protein and the interaction of the concave side of the protein with the lipid membrane of the LD. The 3D structure model of Oil1 protein is most similar to the four-helix bundles, a structure that was also found in perilipins, LD-associated proteins. In summary, our sequence and structure analyses suggested that Oil1 protein consists mainly of amphipathic helices, which might contribute to the binding of the protein to the LD surface, as it was shown for perilipins.

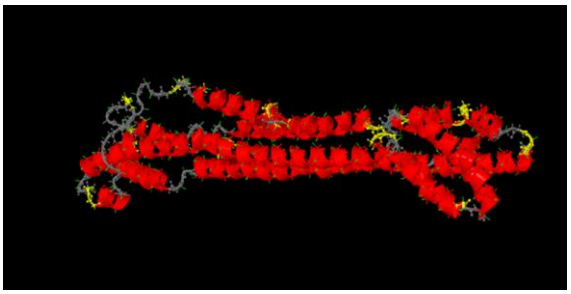

Figure 5. **Three-dimensional model of Oil1 protein**. The 3D structure of the yeast *Y. lipolytica* Oil1 protein was constructed using the I-Tasser server and presented with the Jena3D Viewer. Alpha helices are coloured red, while the unstructured links between them are shown in grey.

Other subjects

In 2018, we also participated at several research projects out of the thematic scope of our department. Two collaborative projects resulted in publications in 2018.

As partners in the study of glioblastoma multiforme (GBM), the most common and lethal form of brain tumour, project led by colleagues from the Medical Faculty of the University of Ljubljana, we participated with the confocal microscopy analysis. To improve the therapy of this tumour and patient outcome, sustained drug delivery to glioma cells is needed, while minimising toxicity to adjacent neurons and glia cells. This may be achieved through an anti-proteomic approach based on nanobodies, the single-domain antigen-binding fragments of heavy-chain antibodies of the camelid adaptive immune system. In this work, we reported on the validation and quantification of a nanobody raised against mitochondrial translation elongation factor (TUFM). Due to its specificity and pronounced inhibitory effect on GBM stem cell growth, we proposed the use of this anti-TUFM nanobody for GBM *in vitro* immunoimaging and potentially also cancer stem cell targeting (N. Samec et al., *Oncotarget*, 9 (2018), 17282–17299).

We also contributed to a study of engineering recombinant *Lactococcus lactis* as a delivery vehicle for BPC-157 peptide with antioxidant activities led by Dr. A. Berlec from the Department of Biotechnology at Jožef Stefan Institute. Our expertise in flow cytometry and dynamic analysis of oxidative stress in mammalian cells was crucial for the determination of antioxidant activity of BPC-157, a pentadecapeptide drug candidate for inflammatory bowel disease. In this study, antioxidant BPC-157 was successfully produced by engineered lactic acid bacteria *Lactococcus lactis*, which may be used in the future as a delivery vehicle for this anti-inflammatory peptide in treatments of gastrointestinal inflammation (K. Škrlec et al., *Appl. Microbiol. Biotechnol.*, 102 (2018), 10103–10117).

Most important publications in 2018

1. Latinović, Z., Leonardi, A., Kovačič, L., Koh, C.Y., Šribar, J., Trampuš Bakija, A., Venkateswarlu D., Kini, R.M. and Križaj, I.: The first intrinsic tenase complex inhibitor with serine protease structure offers a new perspective in anticoagulant therapy. Thromb. Haemost., 118 (2018), 1713–1728
2. Jarc, E., Kump, A., Malavašič, P., Eichmann, T.O., Zimmermann, R. and Petan, T.: Lipid droplets induced by secreted phospholipase A_2 and unsaturated fatty acids protect breast cancer cells from nutrient and lipotoxic stress. Biochim. Biophys. Acta – Mol. Cell Biol. Lipids, 1863 (2018), 247–265
3. Bhutada, G., Kavšček, M., Hofer, F., Gogg-Fassolter, G., Schweiger, M., Darnhofer, B., Kordiš, D., Birner-Gruenberger, R. and Natter, K.: Characterization of a lipid droplet protein from *Yarrowia lipolytica* that is required for its oleaginous phenotype. Biochim. Biophys. Acta – Mol. Cell Biol. Lipids, 1863 (2018), 1193–1205
4. Basu Ball, W., Baker, C.D., Neff, J.K., Apfel, G.L., Lagerborg, K.A., Žun, G., Petrovič, U., Jain, M. and Gohil, V.M.: Ethanolamine ameliorates mitochondrial dysfunction in cardiolipin-deficient yeast cells. J. Biol. Chem., 293 (2018), 10870–10883
5. Gostinčar, C., Zajc, J., Lenassi, M., Plemenitaš, A., de Hoog, S., Al-Hatmi, A.M.S. and Gunde-Cimerman, N.: Fungi between extremotolerance and opportunistic pathogenicity on humans. Fungal Divers., 93 (2018), 195–213