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

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 ($sPLA_2$s) 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, and to develop novel and more effective therapeutic treatments of envenomation.

The molecular mechanism of action of ammodytoxin A (AtxA), a potent β-neurotoxic $sPLA_2$ from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*; *Vaa*), has already been largely described. One of the questions, which still needs some clarification, is the role of the phospholipase activity in β-neurotoxicity. To this end, we prepared enzymatically inactive mutant of AtxA, AtxA(D49S). In collaboration with the research group from the Veterinary Faculty, University of Ljubljana (VF/UL), we published a thorough electrophysiological characterisation of AtxA and AtxA(D49S) (M.C. Žužek et al., *Toxicon* 247 (2024), 107833). Important conclusion of our study was that the effects of AtxA independent of the enzymatic activity cannot be studied with classical electrophysiological measurements on the isolated neuromuscular preparation. Our results also suggested that the inhibition of cytochrome c oxidase activity by AtxA is not involved in the rapid neuromuscular blockade by this β-neurotoxin, but that its pathological consequences are rather long-term.

AtxA has been used as a tool to study the mechanism of regeneration of the nerve terminal after the trauma. In consortium, led by Professor C. Montecucco from the Department of Biomedical Sciences, University of Padua, and the Institute of Neuroscience, National Research Council, Padua, Italy, we published a research paper on the action of an agonist of a G-protein-coupled chemokine receptor CXCR4. We demonstrated that a small molecule agonist of CXCR4, dubbed NUCC-390, induces a rapid regeneration of the motor axon terminal with functional recovery of the neuromuscular junction. Our results qualify NUCC-390 as a promising novel therapeutic agent capable of improving recovery from the paralysis caused by the snakebite of neurotoxic vipers (M. Stazi et al., *Journal of Neurochemistry* 168 (2024), 428–440).

It has been demonstrated that certain $sPLA_2$s specifically bind to nicotinic acetylcholine receptors (nAChRs). The binding of ACh or other agonists, such as nicotine and its derivatives, to nAChRs has been linked to uncontrolled cell division, prevention of apoptosis and induction of angiogenesis, ultimately supporting tumour growth and metastasis. However, antagonists of nAChRs showed opposite effects on the cells, indicating their potential value in cancer therapy. Among the naturally occurring nAChR antagonists, found in various venoms, snake venom $sPLA_2$s were also shown to suppress ACh-elicited ion currents. For this reason, we investigated the anti-cancer effect of an array of human $sPLA_2$s and their single-point enzymatically inactive mutants to assess their lung cancer therapeutic potential. In collaboration with pharmacologists from the University of Leuven, Belgium, we have been determining the effect of these proteins on α7- and muscle-type nAChRs. The most interesting result was obtained with GV(H48Q), which was absolutely selective for α7-nAChR. We used then GV(H48Q) to assess its effects on viability, cytotoxicity, proliferation and apoptosis of various lung cancer cell lines as well as one non-cancerous lung cell line. We demonstrated that GV and GV(H48Q) are able to prevent the ACh-induced cell proliferation and viability. In parallel, we were also involved in a similar study with

another group of α7-nAChR antagonists, 3-alkylpyridinium salts (APS). Two papers describing the effects of APS7 and APS8, either free or packed in gelatine nanoparticles, on human lung cancer cells have been published in 2024 (A. Joukhan et al., *Marine Drugs* 22 (2024), 147; V. Kononenko, et al., *Biomedicine & Pharmacotherapy* 177 (2024), 117007). We published also a review paper on the role of nAChR in cancer (T. Bele et al., *BBA – Molecular Basis of Disease* 1870 (2024), 166875).

We continued the study of snake venom proteins that affect the process of blood coagulation – haemostasis. In the scope of the research project J1-2475, funded by the Slovenian Research and Innovation Agency (ARIS), we have been investigating a unique anticoagulant homologue of a serine protease (SPH) from the venom of the nose-horned viper, VaaSPH-1, in direction of developing completely new and safe drugs with anticoagulant activity. We searched for the best possible conditions for the expression of VaaSPH-1 as well as its binding protein, blood coagulation factor VIIIa (FVIIIa), in mammalian HEK293-F cells. In collaboration with the Faculty of Pharmacy UL, we have been designing low-molecular-mass FIX antagonists. The most promising ones *in vitro* are waiting to be tested for their anticoagulant activity *in vivo*.

We previously demonstrated that the snake venom serine protease VaaSP-VX exhibits a unique enzymatic activity that promotes blood clotting by simultaneously activating both FV and FX. Such an enzyme would be an ideal replacement for dilute Russell's viper venom (dRVV), which is currently used in clinical testing for the detection of lupus anticoagulants (LA test) but lacks sufficient reliability. To establish a standardized clinical LA test using VaaSP-VX, a larger quantity of this protein is required. Recombinant production is necessary to achieve this, and a major challenge is that its sequence is only partially known. One approach to overcoming this limitation was to characterise the enzymatic properties of VaaSP-6, a structurally similar protease that we recently purified from the same venom. Since its full cDNA sequence is available, we are able to produce it recombinantly. Unfortunately, as we have shown, the enzymatic activities of VaaSP-6 and VaaSP-VX differ significantly (K. Požek et al., in preparation). Therefore, our future efforts will focus on obtaining the complete sequence of VaaSP-VX to enable its recombinant production.

In the past year, we also experimentally concluded our ARIS research project J3-2534. In this project, together with colleagues from the Centre for Clinical Toxicology and Pharmacology of the University Medical Centre Ljubljana (UMCL), we investigated an interesting clinical effect observed in patients envenomed by the nose-horned viper, namely a profound, transient and reversible thrombocytopenia of functional platelets. Platelets play a central role in thromboembolic diseases such as myocardial infarction and ischemic stroke. Existing antiplatelet drugs have a common side effect – a reduced number of platelets whose activity is inhibited. This condition carries a high risk of bleeding (haemorrhage), especially in interventional cardiology and angiology that use an antithrombotic approach. Our results could pave the way for the development of a new group of antiplatelet agents that would reduce the risk of dangerous bleeding in interventional cardiology and angiology, and increase the efficacy of vasodilatation and clot removal. We have demonstrated that reversible thrombocytopenia in patients envenomed by *Vaa* is induced by proteins similar to type C lectins (Vaa-snaclecs). We have isolated several snaclecs from the *Vaa* venom and showed that Vaa-snaclec-3/2 in particular induces severe thrombocytopenia through its interaction with the GPIb platelet receptor. In collaboration with our partners from the VF/UL, we have performed an *in vivo* study in a mouse model of arterial thrombosis to validate the potential of Vaa-snaclec-3/2 to prevent clot formation and arterial occlusion after experimentally induced vascular injury, and to determine its potential for medical applications (Figure 1). One part of the results has been published (M. Dobaja Borak et al., *Thrombosis and Haemostasis* 2024, doi: 10.1055/a-2408-9375), the other will follow soon (M.C. Žužek et al., in preparation).

Within the research network, comprising experts from UMCL, University Hospital and University of Split, University of Zagreb (UZ) and our group, we analysed samples of patients who were envenomed by *Vaa* and treated with different antisera. One article has been submitted for

publication in 2024 (M. Dobaja Borak et al., *Thrombosis and Haemostasis,* submitted), the other is still being prepared (T. Kurtović et al., in preparation).

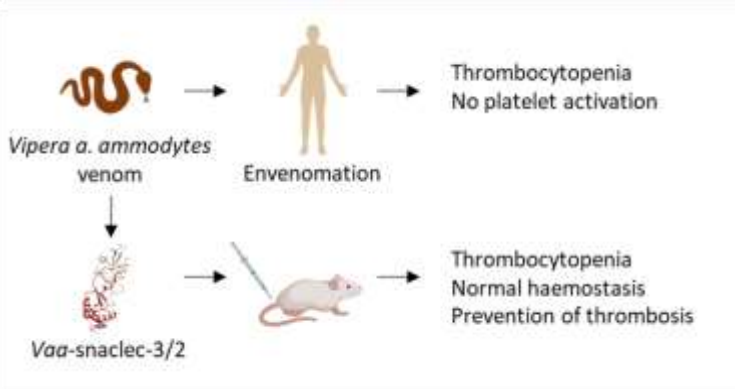

Figure 1*: In our work (M. Dobaja Borak et al., Thrombosis and Haemostasis 2024, doi: 10.1055/a-2408-9375), we demonstrated that the reversible thrombocytopenia of functional platelets upon Vaa envenomation is induced by Vaa-snaclec-3/2. We also showed that Vaa-snaclec-3/2 protected mice from arterial occlusion demonstrating its antithrombotic potential in interventional cardiology.*

Within our large international project on the nose-horned viper genome sequencing and analysis with the Technical University of Denmark and Beijing Genomics Institute, we succeeded to assemble a high-quality *de novo* chromosomal-level genome for this species. We achieved a 131-fold coverage, surpassing previous snake genome assemblies. Our analysis revealed a substantial expansion of olfactory receptor genes, which may be linked to the species' adaptation to high-altitude and cold environments. Furthermore, we identified 112 venom-related genes across 15 families, with notable tandem duplications in snake venom metalloproteases, shedding light on the molecular evolution of its venom. Transposable elements, particularly LINEs, were found abundant, suggesting ongoing genomic activity. This assembly provides crucial insights into the evolutionary dynamics of venomous snakes and offers a valuable resource for comparative genomics, antivenom research, and development of venom-derived therapeutics (W. Rao et al., in preparation).

Our scientific achievements in the field of toxinology were very well recognised also in 2024. Most prestigious was the invitation to I. Križaj to be a keynote lecturer at the 22nd World Congress of the International Society on Toxinology in Singapore (Figure 2). He was also invited as a speaker at the EMBO workshop in Girona, Spain, at the 51st European Muscle Conference in Ljubljana, and at the 32nd Jožef Stefan Days. For his outstanding scientific achievements, I. Križaj received the Jožef Mrak Award in 2024. A. Leonardi received the Outstanding Reviewer Award from the scientific journal *Toxins* for her highly professional contribution.

Figure 2*: Dr. I. Križaj during his keynote talk at the 22nd World Congress of the International Society on Toxinology in Singapore in May 2024.*

Lipid metabolism and signalling

Lipids are vital for life, forming the membranes of all cells and organelles while serving as the most efficient energy storage. However, due to their structural and functional diversity, as well as the complexity of their assemblies, the roles of lipids and lipid metabolism in health and disease remain poorly understood. Lipid droplets are fat storage organelles present in all eukaryotic cells that could help us understand lipid function at the cellular level and in various pathophysiological conditions associated with dysregulated lipid metabolism. These dynamic organelles are involved in essential cellular processes, ranging from energy production and membrane synthesis to infection and inflammation. Our work on the cell and molecular biology of lipids is focused on answering the following questions: (1) How are lipid droplets involved in the generation of lipid signalling molecules? (2) What is the role of the lipid droplets in cellular fatty acid trafficking and the control of membrane function during cellular stress? and (3) How do lipid droplets cooperate with other organelles to fight nutrient stress (Figure 3)?

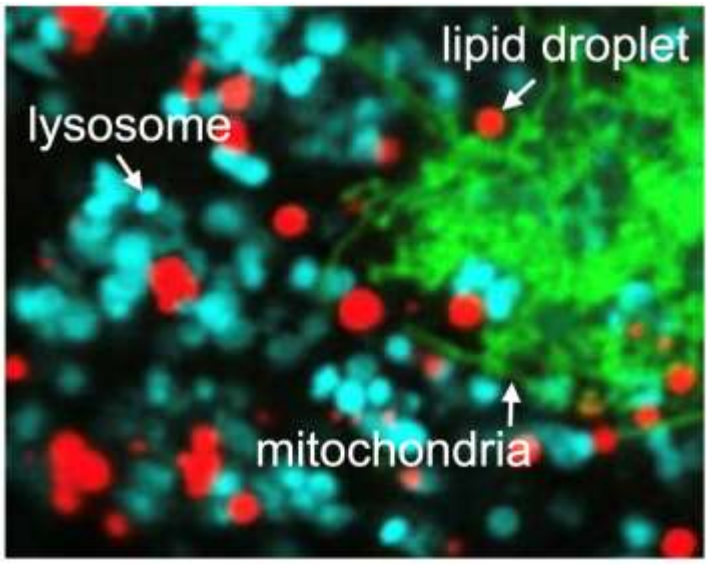

Figure 3*: Lipid droplets work with other organelles to protect cells from severe nutrient deficiency. Using genetic approaches, fluorescent dyes and protein markers we are developing a model system to track organelle interactions and intracellular lipid flux. This system uses live-cell confocal imaging and will allow us to examine some of the key molecular pathways involved in lipid and organelle function in stressed cells. The image the dynamic network of organelles in one cancer cell. Lipid droplets are shown in red, lysosomes in blue and mitochondria in green.*

Following our recent report (E. Jarc Jovičić et al., *Molecular Metabolism* 76 (2023), 101791) on a novel mechanism of fatty acid trafficking between membrane phospholipids and triglycerides, involved in the generation of potent lipid mediators that promote tumor growth and inflammation, we were invited to participate in a community driven review paper on the complexities of lipid mediator biology and their clinical use. This work, entitled “Oxylipin profiling for clinical research: Current status and future perspectives”, was published in one of the leading journals in this field (K. Parchem et al., *Progress in Lipid Research* 95 (2024), 101276). Oxylipins are lipid signaling molecules that are formed by oxidation of fatty acids and participate in essential processes, such as cell growth, inflammation, pain and immunity. In this review, we provided an updated and key information on the metabolism of oxylipins, their different forms in the systemic circulation, the current limitations in deducing oxylipin cellular effects from *in vitro* studies, as well as the biological and technical confounding factors required for a proper interpretation of oxylipin profiles.

Our ongoing research on lipid droplets and fatty acid fluxes in ferroptotic cell death is at the forefront of the field, leading to several invited talks at renowned conferences and institutions. These include the annual meeting of ASBMB (the American Society for Biochemistry and Molecular Biology) in San Antonio, TX, USA; the Nencki Conference for Life Sciences in Warsaw, Poland; the Lipids and Cell Plasticity Symposium in Aveiro, Portugal; and an invited seminar at the Guest Lecture Series, Lipid Lipolysis SFB, at Medizinische Universität Graz in Graz, Austria.

High-throughput genetics and functional genomics in yeast *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* is a well-established model organism for basic research, and a cell factory in biotechnology. In biotechnological applications, it is also important for synthetic biology given its highly efficient homology recombination-based assembly of DNA fragments.

We successfully finished the ARIS project L4-3181 ('Hierarchical DNA assembly for advanced applications in biopharmaceuticals production and cell therapy'), whereby we developed a technology for the production of genomes of recombinant bacteriophages with modified host-range. The technology has been transferred to the industrial partner and co-financer of this project. The developed methodology within this project will enable us to use combinatorial genetics approaches in our future research (Figure 4).

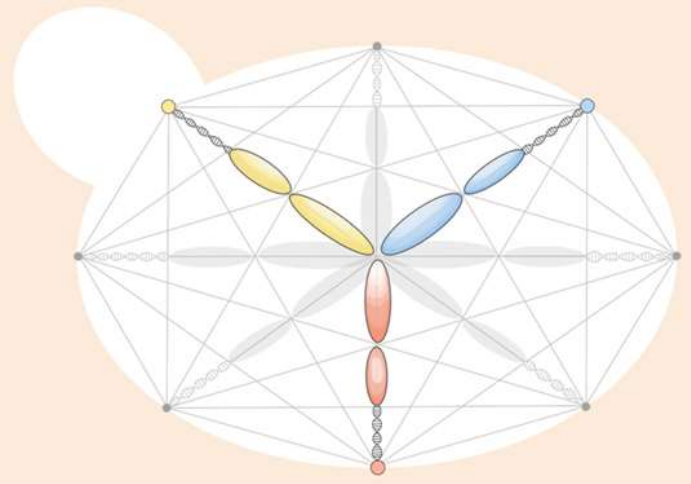

***Figure 4**: Schematic illustration of combinatorial genetics in yeast Saccharomyces cerevisiae (author: P. Kobal).*

Our B-Yeast technology, which is based on combinatorial genetics approaches developed in this project, has been awarded as the Best Innovation with commercial potential in 2024 at the 17th International Technology Transfer Conference, held at the Jožef Stefan Institute (JSI) (Figure 5).

***Figure 5**: The yeast team in the lab, and the JSI Best Innovation with commercial potential in 2024 award.*

We also successfully finished the ERACoBioTech project OLEOFERM (https://oleoferm.eu/), whereby we published a research paper about high-throughput screening of non-conventional yeasts for conversion of organic waste to microbial oils via carboxylate platform (M. Žganjar et al., *Scientific Reports* 14 (2024), 14233). We have finished the genomics and transcriptomics

analyses of the identified biotechnologically useful strain of the oleaginous yeast species *Yarrowia lipolytica* with a promising potential for lipid production from short-chain fatty acids, and the results are planned to be published in 2025.

Within the ARIS project J4-4560, *Engineering of polygenic traits in S. cerevisiae*, we have generated an outbred population of yeast strains based on parental strains from 23 different clades. We devised an approach to identify the most thermotolerant segregants from this population.

We presented the results on our developed CRISPR-dCAS technology to modulate gene expression through histone acetylation in the yeast *Saccharomyces cerevisiae* at the Congress of the Genetics Society of Slovenia.

Evolutionary genomics

In the field of research on protein superfamilies, in collaboration with V. Stoka from the JSI Department of Biochemistry and Molecular and Structural Biology (B1), we investigated the origin and evolution of diverse cathepsin peptidases, from aspartate (cathepsin D and cathepsin E), serine (cathepsin A and cathepsin G) and cysteine (cruzipains and cathepsin F in animals) peptidases. We also investigated the origin and evolution of various cysteine peptidases inhibitors (from bifunctional cystatins, multistefins, and inhibitor families I31, I42, I71 and I81). In addition, we analysed cysteine peptidases and their inhibitors in eukaryotic pathogens. We discovered a new group of C1A cysteine peptidases with an I42 domain in many bacteria, some archaea and only in one eukaryote, euglenid (*Rhabdomonas*) – in this group of peptidases we found several cases of horizontal gene transfer. In insects we found the largest cathepsin F, which is about 5000 amino acids long, and this length is a consequence of the extremely increased number of N-terminal cystatin domains. Discovery of previously unknown multistefins in different eukaryotes was also interesting, we found that the number of stefin domains is variable and consists from 2 to 15 repeats of the stefin domain (V. Stoka et al., in preparation).

In the field of research on the olm (*Proteus anguinus*) genome, we searched for important genes for additional analyses using CRISPR or gene knockout among the numerous genes that evolve under positive selection. We determined which of these genes are hub genes, essential genes or disease genes. With the help of this analysis, we significantly reduced the number of important genes for additional analyses using CRISPR or gene knockout (R. Kostanjšek et al., in preparation).

In basal metazoans, we found new pore-forming proteins from the aerolysin superfamily in the new protein database (LukProt database) and analysed their domain architectures (D. Kordiš, in preparation).

Other subjects

In 2024, we also participated in different projects outside the thematic scope of our department. Only the projects for which publications have been published or are in preparation are listed below.

Our expertise in cancer cell biology was instrumental in our collaboration on the ARIS project J2-3040 lead by the colleagues from the JSI Department for Materials Synthesis (K8). Together, we developed a new approach for the delivery and actuation of individual magnetic nanoparticles with enhanced potency in killing cancer cells (T. Goršak et al., *Journal of Colloid and Interface Science* 657 (2024), 778–787).

We were invited to join our colleagues from the JSI Department of Gaseous Electronics (F6), to study the reduction of antigenicity of common ragweed pollen and its primary allergen Amb a 1 with cold atmospheric pressure air plasma (N. Hojnik et al., *Journal of Hazardous Materials* 479 (2024), 135640).

With structural analysis we helped our colleagues from the University of Campania, Caserta, Italy to characterise sodin 5 as a homologue of type 1 polynucleotide:adenosine glycosylase, commonly known as ribosome-inactivating protein. In the paper, we showed that antifungal activity

of sodin 5 may be biotechnologically very interesting (M. Novak Babič et al., *Biomolecules* 14 (2024), 336).

We participated at the work lead by our colleagues from the University of Belgrade in characterisation of the novel endopolygalacturonase II from *Aspergillus tubingensis* to be used in the fruit juice industry for enhanced liquefaction, juice clarification, and augmentation of its antioxidant potential (M. Pavlović et al., *Food & Function* 15 (2024), 2906–2919).

Colleagues from the Ruđer Bošković Institute and UZ were assisted in researching the mechanism of formation and morphogenesis of biomineral nanostructures of the *Archa noae* shell. We performed structural identification of protein components of the shell that are potentially involved in the biomineralization process (I. Sondi et al., *ACS Biomaterials Science & Engineering*, in press).

We came to the aid to our colleagues from the Medical Faculty UL performing the confocal microscopic analysis for the functional validation of an α-FREM2 nanobody as a molecular tool for targeting specifically glioblastoma stem cells (G. Krapež et al., *Antibodies*, in press).

In the collaboration, led by our colleagues from the JSI Department for Nanostructured Materials (K7), we investigated the potential of vesicles from red blood cell membranes as a safe and efficient delivery system for therapeutic nucleic acids (G. Della Pelle et al., *International Journal of Nanomedicine*, in press).

In collaboration with the group from the Biotechnical Faculty UL, we participated in investigation of the effects of microplastics in soil on the terrestrial crustacean woodlice (*Porcellio scaber*). In the search for molecular markers of microplastic-induced toxicity, we analysed changes in the proteome of the haemolymph of *P. scaber* using mass spectrometry proteomics (A. Leonardi et al., in preparation).

In the study led by researchers from the Faculty of Electrical Engineering UL, we analysed the protein corona composition of different types of nanoparticles using a proteomic approach to specify the binding proteins from human serum (L. Peternel et al., in preparation).

Some outstanding publications in 2024

1. Dobaja-Borak, M., Leonardi, A., Požek, K., Reberšek, K., Podgornik, H., Pirnat, A., Trampuš Bakija, A., Kranjc Brezar, S., Trobec, T., Žužek, M., Frangež, R., Brvar, M. and Križaj, I.: Reversible thrombocytopenia of functional platelets after nose-horned viper envenomation is induced by a snaclec. Thrombosis and Haemostasis (2024), doi: 10.1055/a-2408-9375
2. Stazi, M., Megighian, A., D'Este, G., Negro, S., Ivanušec, A., Lonati, D., Pirazzini, M., Križaj, I. and Montecucco, C.: An agonist of CXCR4 induces a rapid recovery from the neurotoxic effects of *Vipera ammodytes* and *Vipera aspis* venoms. Journal of Neurochemistry, 168 (2024), 428–440
3. Bele, T., Turk, T. and Križaj, I.: Nicotinic acetylcholine receptors in cancer: limitations and prospects. Biochim. Biophys. Acta – Molecular Basis of Disease 1870 (2024), 166875.
4. Žganjar, M., Ogrizović, M., Matul, M., Čadež, N., Gunde-Cimerman, N., González-Fernández, C., Gostinčar, C., Tomás-Pejó, E. and Petrovič, U.: High-throughput screening of non-conventional yeasts for conversion of organic waste to microbial oils via carboxylate platform. Scientific Reports 14 (2024), 14233
5. Žužek, M.C., Ivanušec, A., Herman, J., Šribar, J., Leonardi, A., Frangež, R. and Križaj, I.: Comparative electrophysiological characterization of ammodytoxin A, a β-neurotoxin from the nose-horned viper venom, and its enzymatically inactive mutant. Toxicon 247 (2024), 107833.