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 most important 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 interested in their presynaptic neurotoxicity. The knowledge that we gather by studying toxic $sPLA_2$s represents valuable assistance in understanding 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 A (AtxA) is a neurotoxic $sPLA_2$ from the venom of the nose-horned viper (*Vipera a. ammodytes*). The result of action of this toxin on the motor neuron is the inhibition of secretion of the neurotransmitter acetylcholine into the synaptic cleft and the flaccid paralysis of muscle. Among characteristic effects of AtxA on nerve cells are damaged mitochondria. That the toxin possesses a specific protein receptor in the mitochondria we have reported already in 1998, but untill recently its nature has been escaping the identification. In 2017 we finally succeded to obtain the results that without a doubt confirmed the identity of this protein. It is known that endogenous GIIA $sPLA_2$, very similar in structure to Atx, is localized in mitochondria of mammalian cells. Besides the confirmed involvement of this enzyme in neuritogenesis, its role in this orgalelle is still quite hypothetical and unknown. Based on its similarity to AtxA we suggest the involvement of this enzyme in the regulation of cellular respiration. Deregulation of its function, induced by the increase of its concentration and concomitantly the activity, is likely associated with etiology of some neurodegenerative diseases, such as Alzheimer's, at which the mitochondrial disfunctions are very similar to those inflicted by AtxA at its poisoning the nerve cell. We initiated preparation of a paper describing the first intracellular membrane $sPLA_2$ receptor and we expect an attention-grabbing publication.

In 2017 we continued with the systematic analysis of the components of the nose-horned viper venom. We deepened the proteomic analysis of the venom and expanded it with a comprehensive study of the viper venom gland transcriptome. The transcriptomic survey of *Vipera a. ammodytes* venom glands has shown that the most abundunt venom transcripts (more than a quarter of all) are those encoding precursors of multiple bioactive peptides, i.e. tripeptide metalloproteinase inhibitors, bradykinin-potentiating peptides and natriuretic peptide. They are followed by those of snake C-type lectin-like proteins (Snaclecs), serine proteinases, metalloproteinases of P-II and P-III classes, toxic and nontoxic $sPLA_2$s, and disintegrins. Almost nine tenths of the entire viper transcriptome thus contain the information for these major seven protein groups. The remaining portion of transcripts is that encoding two serine proteinase inhibitors, vascular endothelial growth factor, cysteine-rich protein (Crisp), *L*-aminoacid oxidase and venom nerve growth factor. We also found a few identical transcripts of a novel, so far unidentified protein, rich in leucine residues, whose function remains unknown. A publication with the most complete description of the composition of *Vipera a. ammodytes* venom glands is under preparation. It should significantly improve the planning of therapeutic strategies.

In the scope of systematic analysis of the *Vipera a. ammodytes* venom goes also the identification of a cardiotoxic component of this venom. In collaboration with colleagues from the Clinical Department of Infectious Diseases, University Hospital Centre Split, Croatia, and the Department of Pharmacology, Mostar University School of Medicine, Bosnia and Herzegovina,

we identified the venom component with the strongest effect on heart. In publication we described in detail its effects on the isolated rat heart (S. Karabuva et al., *Toxicon*, 139 (2017), 94–100).

With intensity we studied the nose-horned viper venom proteins that affect the blood coagulation process—haemostasis, in particular two such proteins, a homologue of serine protease with anticoagulant activity (VaaSPH-1) and a serine protease with procoagulant, FVIIa-like activity (SP-10). In collaboration with the group of Dr. Manjunatha R. Kini, the renowned expert for haemostasis from the National University of Singapore, we concluded in the past year a detail characterization of the molecular mechanism of action of both proteins. We developed also a procedure to produce the recombinant form of VaaSPH-1 in mammalian cells. We prepared the publication describing the characteristics of the mode of anticoagulant action of VaaSPH-1, the protein very interesting for the development of a new inhibitor for blood coagulation that specifically affects the intrinsic coagulation pathway. Presentation of this work in the form of poster presentation at the 12th Meeting of the Slovenian Biochemical Society with International Participation in September 2017 on Bled was awarded the 1st prize by the Scientific Committee (Figure 1, Z. Latinović).

Figure 1: Young researchers from our Department were awarded for their work. Our young researchers, Zorica Latinović (left) and Eva Jarc (right), received 1st and 2nd prize for their poster presentations at the 12th Meeting of the Slovenian Biochemical Society with International Participation that took part in Bled between 20th and 23rd Septemer 2017. The Scientific Committee of the conference awarded Zorica with the 1st prize for the work entitled »Serine protease homologue from the venom of the nose-horned viper is a promising new anticoagulant lead molecule« and Eva with the 2nd prize for the work entitled »Lipid droplets are involved in eicosanoid generation and protection against nutrient stress in breast cancer cells«.

We wrote the first article about disintegrins from the nose-horned viper (Z. Latinović et al., *Acta Chim. Slov.*, 64 (2017), 555–559). Disintegrins are polypeptides that bind to integrin molecules and impair in this way their function. The nose-horned viper disintegrins efficiently prevent migration and thus spreading of cancer cells. They thus express an anti-metastatic potential, which gives a good prospective for their development in the direction of a new anti-cancer drug.

In 2017 we continued and concluded the Slovenian-Croatian bilateral research project. The result of a common work with colleagues immunologists from the University of Zagreb and medical doctors from the Centre for Clinical Toxicology and Pharmacology, University Medical Centre Ljubljana is publication of the paper (M. Brvar et al., *Clin. Toxicol.*, 55 (2017), 241–248), in which we described treatment of patients, envenomed by the nose-horned viper venom, using antivenom directed towards the common adder venom (ViperaTAb®). We found out that the treatment using paraspecific antivenom alleviated swelling and temporarily improved systemic

effects of envenomation, by lowering concentration of toxic components in the patients' blood, but did not abolish neurotoxic effects.

On the specific area of toxinology, we collaborated with our colleagues from the Department of Surface Engineering and Optoelectronics (F4) at the Jožef Stefan Institute. Together, we prepared a review paper on mycotoxin decontamination of food and feed comparing new approaches using cold atmospheric pressure plasma decontamination with the "classic" decontamination methods (N. Hojnik et al., Toxins, 9 (2017), 151).

In the past year we invested a lot of energy also in popularization of our scientific activity by presenting our work to the broader community. We presented our activities in the interview dedicated to animal venoms for the Italian RAI, Radio Trieste A, in the emission called Hevreka and in the interview for Ognjišče magazine (I. Križaj, *Ognjišče*, 53 (2017), 102–103).

Springer publishing company invited us to prepare a chapter for their monograph Snake Venoms. The book has been issued in 2017 (D. Kordiš and I. Križaj, Handbook on Toxinology, Springer (2015), ISBN: 78-94-007-6648-8). Amongst other things, we presented in the article a critical overview of all important results on the action of presynaptically neurotoxic $sPLA_2$s, and based on them proposed a hypothesis about the mechanism of action of these toxins and suggested experimental approaches to test it.

As experts from the field of toxinology, we have been invited as lecturers on expert meetings and scientific conferences. Most worth mentioning is the invitation to deliver the keynote lecture at the 19th World Congress of the International Society on Toxinology in Haikou, China, to I. Križaj.

Lipid metabolism and signalization

Lipid droplets are newly recognized organelles composed of a core of neutral lipids, mainly triacylglycerol and cholesterol esters, and covered with a phospholipid monolayer and lipid droplet-associated proteins. They are present in all cells and act as platforms integrating cellular lipid metabolism and signalling, protein management and quality control, viral replication and immunity. Their biogenesis is generally induced when cells are exposed to a surplus of lipids in the environment (E. Guštin et al., *Acta Chim. Slov.*, 64 (2017), 549–554), but, intriguingly, they are also formed in different stressful conditions for the cell, including nutrient deprivation, hypoxia and oxidative stress. Recent studies have revealed that they also accumulate in cancer cells. Given that the ability of cancer cells to survive stress is indispensable for tumour growth and metastasis, lipid droplets may be important for their resistance to various stresses and thus promote tumourigenesis. In our study (E. Jarc et al., *Biochim. Biophys. Acta*, in press), we examined the role of lipid droplets in the protection of aggressive breast cancer cells from lipotoxic and nutrient deprivation-induced stress. We found that cancer cells sequester unsaturated fatty acids from their environment and store them in the form of triglycerides in lipid droplets, which in turn provide fuel for mitochondrial energy production during nutrient deprivation. However, exposing cells to high concentrations of polyunsaturated fatty acids (PUFAs) is toxic to the cells (lipotoxicity), because surplus PUFAs are released from lipid droplets and cause oxidative damage (Figure 2). By silencing the crucial enzyme in 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 these organelles protect sensitive PUFAs 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. Lipid droplets thus balance unsaturated fatty acid trafficking with cell survival mechanisms and protect cancer cells from nutrient and oxidative stress. Our study reveals that targeting lipid droplet metabolism may be exploited to significantly reduce the resilience of cancer cells to oxidative and metabolic stress and thus impair tumour progression. Presentation of this work in the form of poster presentation at the 12th Meeting of the Slovenian

Biochemical Society with International Participation in September 2017 on Bled was awarded the 2nd prize by the Scientific Committee (Figure 1, E. Jarc).

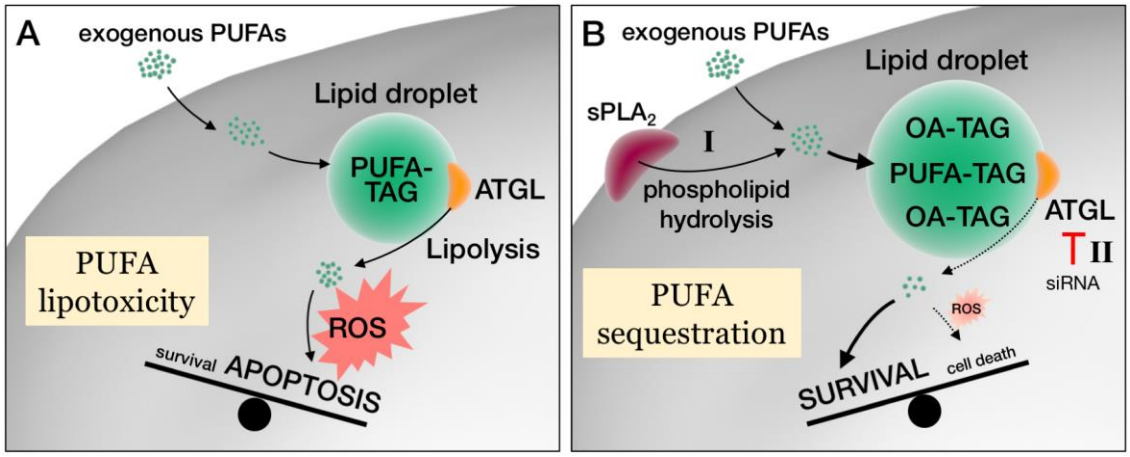

Figure 2: Sequestration of polyunsaturated fatty acids (PUFAs) in lipid droplets protects cancer cells from lipotoxicity. Exposing breast cancer cells to high concentrations of PUFAs leads to the biogenesis of lipid droplets that contain highly unsaturated PUFA-TAG species. The breakdown of lipid droplets by the lipase ATGL leads to the production of reactive oxygen species (ROS) and oxidative stress-dependent apoptotic cell death (A). We found that PUFA lipotoxicity may be reduced by two complementary mechanisms that sequester PUFAs in lipid droplets (B): (I) Cell membrane hydrolysis by the $sPLA_2$ enzyme leading to lipid droplet biogenesis and TAG remodelling (including the incorporation of the monounsaturated oleic acid (OA)) thereby reducing the fraction of highly unsaturated PUFA-TAGs, or (II) inhibition of ATGL-mediated lipolysis leading to retention of PUFAs within lipid droplets. Figure adapted from Jarc et al., *Biochim. Biophys. Acta* (in press).

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

Polygenic trait analysis is one of the fastest developing fields in genetics. It will fundamentally influence our understanding of processes in biotechnology and biomedicine. Using the approaches that we developed ourselves, in which CRISPR/Cas9 method is used to edit the yeast genome at will, we further elucidated genetic architecture of traits connected to neutral lipid content in yeast (Figure 3). The same approaches were used in the studies on the *MKT1* gene, a model gene for several polygenic traits in yeast.

Also obesity in humans and in mice is a polygenic trait. Using yeast as a model organism we studied molecular function of the *TUM1* gene, the homologue of the mammalian gene *TST* which has been shown to be involved in the onset of obesity in humans and mice. We showed that the TUM1 protein is involved in the sterol ester metabolism, but that its function is not identical to the one of the TST protein (K. Uršič et al., *BMC Microbiol.*, 17 (2017), 181).

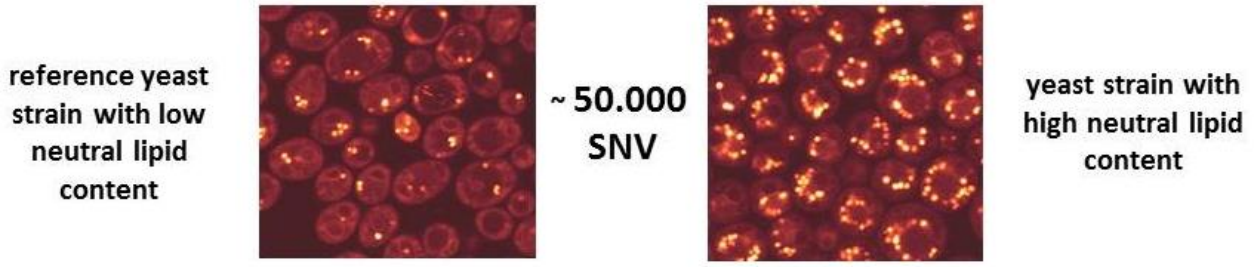

Figure 3: Fluorescent microscopy of strains with low and high neutral lipid content that differ in approximately 50,000 nucleotides over the genome. Polygenic trait analysis methods enable us to find from this set the few that are causal for a given trait.

Analysis of genomes

In a book chapter we have presented the most exciting insights obtained from our study about the origin, distribution, diversity, and evolution of retroelement-derived domesticated genes in mammals (D. Kordiš, Evolutionary Biology: Self/Nonself Evolution, Species and Complex Traits Evolution, Methods and Concepts, Springer (2017), ISBN: 978-3-319-61569-1). We have mapped the life history of domesticated genes, from birth, their fixation in the genome, gain of regulatory elements and structural complexity to complete integration into the functional network of the cell. We have demonstrated that domesticated genes originated from retroelement remains and that they acquired regulatory regions *de novo*. Newly emerged domesticated genes may evolve new functional roles through adaptive evolution of encoded proteins and/or by developing new spatial or temporal expression patterns. The regulatory wiring of domesticated genes and their rapid fixation in the ancestor of placental mammals have played an important role in the origin of their innovations and adaptations, such as placenta and newly evolved brain functions. We have demonstrated the utility of molecular domestication as a good model for understanding the origination and functional evolution of novel genes.

Early evolutionary analyses of $sPLA_2$ toxins in venomous animals took place in the “pre-genomic era”, and were based on a small sample of taxonomic diversity and diversity within the $sPLA_2$ toxins. Since then, the number of representatives has increased significantly, largely due to the accumulation of the venom transcriptomic resources since the large genomic data regarding $sPLA_2$ toxins in venomous animals are still very sparse. In the book chapter (D. Kordiš and I. Križaj, Handbook on Toxinology, Springer (2017), ISBN: 978-94-007-6409-5) we highlighted how the progress in the last decade has increased our understanding of the evolution of $sPLA_2$ toxins in venomous animals.

TUM1 is the yeast *Saccharomyces cerevisiae* ortholog of the human *TST* gene. TUM1 and TST/MPST (mercaptopyruvate sulphurtransferase) proteins belong to the rhodanese protein superfamily. Our analysis has demonstrated that rhodanese superfamily is widespread in archaea, bacteria and in all major eukaryotic groups (K. Uršič et al., *BMC Microbiology*, 17 (2017), 181). The prokaryotic proteins are scattered among the eukaryotic representatives, indicating the possibility of horizontal gene transfer, which is further supported by relatively high levels of sequence identity between some prokaryotic and eukaryotic rhodanese representatives (Figure 4). Ancestral states of the *TST* and *MPST* chromosomal positions were reconstructed from comparisons of syntenic positions between the diverse vertebrate lineages. Analysis of conserved synteny has demonstrated that the gene duplication of rhodanese gene superfamily occurred in the ancestor of land vertebrates (~ 359 Mya), producing *TST* and *MPST* genes.

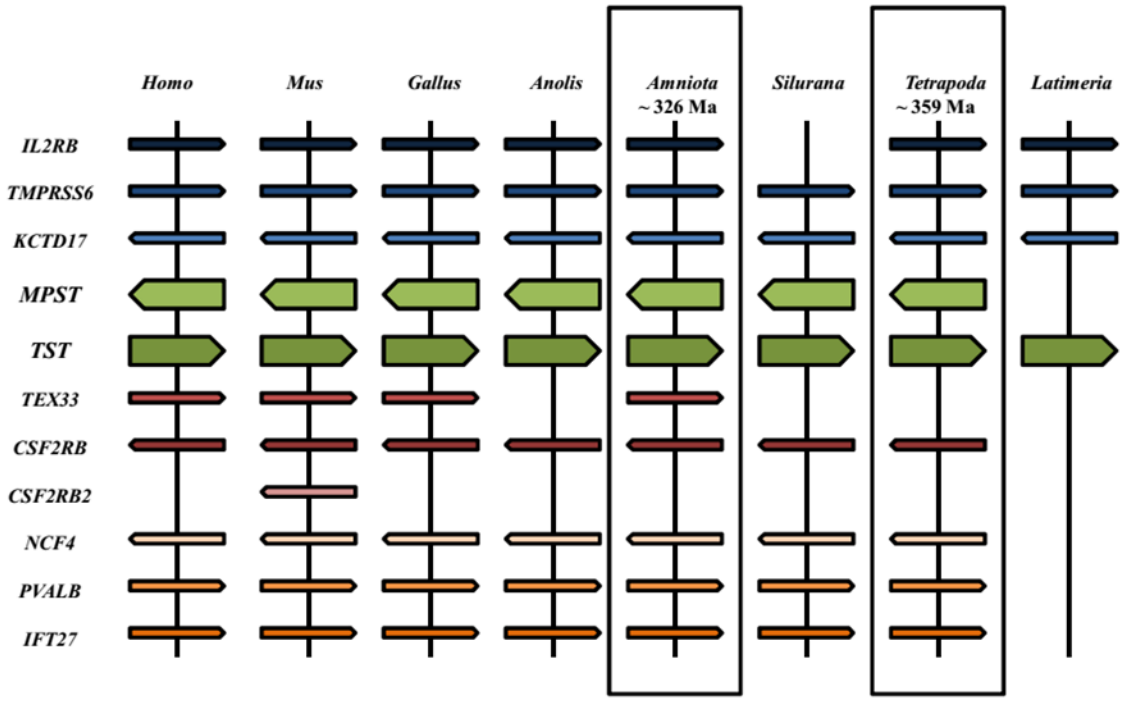

Figure 4: Conserved synteny of the rhodanese superfamily in vertebrates. Chromosomal regions carrying *TST* and *MPST* genes in the species considered in this analysis were compared, and neighbouring genes with conserved synteny were identified. Horizontal lines denote orthologous relationships. Each gene is represented by a horizontal line on the chromosome. Neighbouring genes that are in synteny are shown with a schematic indication of their orientation and distance (not to scale). Ancestral states of the *TST* and *MPST* chromosomal positions in *Amniota* and *Tetrapoda* were reconstructed from comparisons of syntenic positions between multiple vertebrate lineages. Analysis of conserved synteny demonstrated that the gene duplication of rhodanese gene superfamily occurred in the ancestor of land vertebrates (~ 359 Mya). The figure is reproduced from K. Uršič et al., *BMC Microbiology*, 17 (2017), 181.

Other subjects

In 2017 we also participated at several research projects out of the thematic scope of our department.

As partners on the project led by colleagues from the Faculty of Electrical Engineering of the University of Ljubljana (UL) we accomplished structural identification analysis of protein corona composition of nanoparticles prepared in different dispersion media. As protein corona of nanoparticles primarily determines the pathophysiological characteristics of nanoparticles in biological systems, the knowledge about its controlled formation is vitally important for the safe use of nanoparticles in medicine. Our results and conclusions we published in 2017 (K. Strojan et al., *PLoS One*, 12 (2017), e0169552).

Also in the field of nanoparticles research, we were partners in collaboration coordinated by our colleagues from the Biotechnical Faculty UL. We participated with the cell culture studies, determining the influence of non-cytotoxic concentrations of silica-coated superparamagnetic iron oxide nanoparticles (SiO_2-SPIONs) on human alveolar epithelial A549 cells, a model of alveolar type II cells. The pulmonary delivery of nanoparticles is namely a promising approach in

nanomedicine. We succeeded to publish our results in the prestigous journal Nanotoxicology (V. Kononenko et al., *Nanotoxicol.*, 11 (2017), 419–429).

On the project led by the partners from the Ruđer Bošković Institute in Zagreb we participated at establishing the mechanism of formation and morphogenesis of the aragonite nanostructure of the common cuttlefish (*Sepia officinalis*) cuttlebone. We accomplished the mass-spectrometric identification of protein components of the cuttlebone, potentially involved in the process of biomineralization *i.e.* initiation of the extracellular nucleation of aragonite nanocrystals. The work has already been published (V. Čadež et al., *J. Coll. Interf. Sci.*, 508 (2017), 95–104).

We collaborated also with our colleagues from the NMR Centre of the Utrecht University, the Netherlands, at establishing the mechanism of binding of structure-specific endonuclease ERCC1/XPF on DNA in the process of its repair (D. Das et al., *J. Biol. Chem.*, 292 (2017), 2842–2853).

Most important publications in 2017

1. Karabuva, S., Lukšić, B., Brizić, I., Latinović, L., Leonardi, A. and Križaj, I.: Ammodytin L is the main cardiotoxic component of the *Vipera ammodytes ammodytes* venom. Toxicon, 139 (2017), 94–100
2. Kordiš, D. and Križaj, I.: Secreted phospholipases A_2 with β-neurotoxic activity. In: Handbook on Toxinology (Gopalakrishnakone, P., Inagaki, H., Mukherjee, A.K., Rahmy, T.R. and Vogel C.-W.; Eds.), Volume: Snake Venoms. ISBN: 978-94-007-6409-5 (2017), Springer; pp. 67–86
3. Uršič, K., Ogrizović, M., Kordiš, D., Natter, K. and Petrovič, U.: TUM1 is involved in the metabolism of sterol esters in *Saccharomyces cerevisiae*. BMC Microbiol., 17 (2017), 181
4. Kononenko, V., Erman, A., Petan, T., Križaj, I., Kralj, S., Makovec, D. and Drobne, D.: Harmful at non-cytotoxic concentrations: SiO_2-SPIONs affect surfactant metabolism and lamellar body biogenesis in A549 human alveolar epithelial cells. Nanotoxicology, 11 (2017), 419–429
5. Strojan, K., Leonardi, A., Bregar, V.B., Križaj, I., Svete, J. and Pavlin, M.: Dispersion of nanoparticles in different media importantly determines the composition of their protein corona. PLoS One, 12 (2017), e0169552