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 which we gather by studying toxic $sPLA_2$s represents valuable assistance at understanding 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 ammodytes ammodytes*). In 2016 we continued with the study of a role of the phospholipase activity of this molecule in neurotoxicity. We produced large amount of a recombinant, enzymatically inactive form of AtxA, and characterized it.

We studied the process of AtxA neurotoxicity using PC12 model cell line. Mitochondria are the organelles which are most severely affected by AtxA in nerve cells. We discovered that AtxA binds specifically to R25 in mitochondria. We succeeded to demonstrate the interaction between these two proteins also *in vivo* in these cells. Moreover, we showed that AtxA, as also its enzymatically inactive mutant, inhibited the activity of this protein in mitochondria isolated from PC12 cells. We also checked the influence of both $sPLA_2$ molecules on the mitochondrial membrane potential. While AtxA induced its collapse, enzymatically inactive mutant of AtxA did not have any effect. The influence on the mitochondrial membrane potential is therefore, contrary to the influence on R25, dependent on phospholipase activity. The endogenous $sPLA_2$, of the same structural type (group IIA) as AtxA, has been found in mitochondria. Its physiological role in this organelle is still unknown. Our results suggest that this enzyme, through its interaction with R25, may be involved in regulation of cellular respiration. Consequently, deregulation of its function may result in different neurodegenerative diseases, characterized by malfunction of mitochondria.

In 2016 we continued with the venom proteomics or venomics, the systematic analysis of the components of the nose-horned viper (*Vipera ammodytes ammodytes*) and the common adder (*Vipera berus berus*) venoms. The first comprehensive proteomic results of the common adder venom, very important for preparation of optimal therapeutic strategy upon envenomation (Figure 1), we succeeded to publish in respected *Journal of Proteomics* (Z. Latinović et al., *J. Proteomics*, 146 (2016), 34–47).

In the past year we also worked intensively on the preparation of a report on the most, to date, inclusive analysis of the nose-horned viper venom that is scheduled for publication in 2017. In the scope of systematic analysis of the *Vipera a. ammodytes* venom goes also the identification of 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 performed and published the analysis of effects of all venom fractions on the isolated rat heart (S. Karabuva et al., *Toxicon*, 121 (2016), 98–104). The most prominent effect on the heart was obtained with the venom fraction containing $sPLA_2$. We will prepare all the components of this venom fraction in a pure form to define those that are cardiotoxic and describe details of their interference with heart rate.

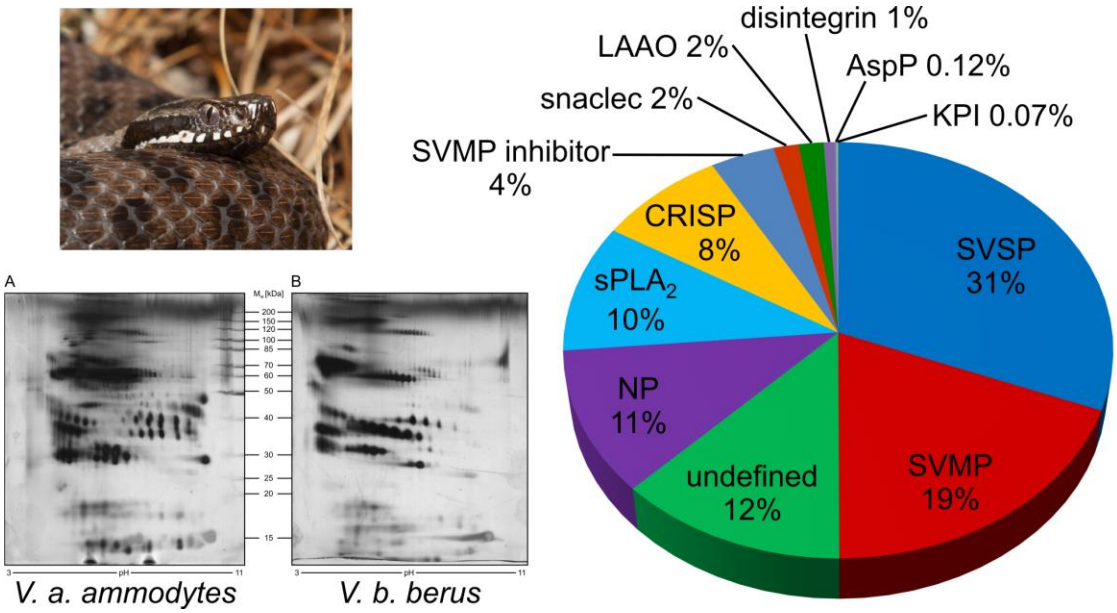

Figure 1: Comparative venomics of two medically most important European vipers. We have analysed the venom proteome of *Vipera berus berus* (*V.b.berus*) (right part of the figure), the most widely distributed venomous snake in Europe (upper left part of the figure), and compared it with the venom proteome of the most venomous viper in Europe, *Vipera ammodytes ammodytes* (*V.a.ammodytes*) also using two-dimensional gel electrophoresis (lower left part of the figure). Obtained results provided an explanation for the effectiveness of treatment of *V.b.berus* envenomation by *V.a.ammodytes* antiserum and explained why full protection of *V.a.ammodytes* venom poisoning by *V.b.berus* antiserum should not always be expected.

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 the past year both molecules were extensively characterized. We determined the complete cDNA sequence of VaaSPH-1. Consequently, we also know its complete amino acid sequence. We started to develop a procedure to produce this protein in mammalian cells. In the case of SP-10, we still do not know its complete cDNA and protein structures. In collaboration with the group of Dr. Manjunatha R. Kini, the renowned expert for haemostasis from the National University of Singapore, we made in 2016 a big step forward in analysing the molecular mechanisms of VaaSPH-1 and SP-10. Both molecules have special properties and are therefore very interesting for further development in a direction of medical usage: VaaSPH-1, as a strong inhibitor of blood coagulation without an enzymatic activity, and SP-10, as the first snake venom serine protease that specifically activates FX.

We wrote the first article about disintegrins from the nose-horned viper, which is now in the revision process. 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.

Following an arrest, in 2016 we continued the study of snake venom cysteine-rich secretory proteins (CRISPs). Snake venom CRISP molecules can cause paralysis of peripheral smooth muscles and hypothermia by inhibiting certain types of ion channels. Physiological effect of the nose-horned viper CRISPs is still unknown and we intend to describe it.

In 2016 we initiated 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 (UMC) is publication of two papers. In the first, we described the efficiency of commercial antivenom directed towards European viperids (Viperfav^{TM}) on a real patient. The key conclusion of this

work is that the dose of Viperfav™, recommended for treatment of persons envenomed by the venom of *Vipera aspis* or *Vipera berus berus*, is probably not enough in the case of a serious envenomation by the venom of *Vipera ammodytes ammodytes* (T. Kurtović et al., *Toxins*, 8 (2016), 244). For this reason, we recommended constant monitoring of such patients during antivenom treatment. In the second paper, which is accepted for publication (M. Brvar et al., *Clinical Toxicology*, in press), we describe 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.

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. Invited by the SATENA society, I. Križaj presented a public lecture in the scope of the action "Science on Street". The lecture entitled "Animal Venoms: from Deadly to the Healthy" was very well attended and received an excellent response (Figure 2). The lecture was recorded and is available at Videolectures.net (http://videolectures.net/znanostnacesti_krizaj_zivalski_strupi/). Further, we participated at the preparation and presentation of a popular scientific TV broadcast "Bite the Science" entitled "Animal Venoms – Deadly and Useful" (http://4d.rtvslo.si/arhiv/ugriznimo-znanost/174441881). As well, we presented toxinology and our achievements in this field of science in two interviews: for the national radio in an emission "Frequency X" on animal venoms (http://4d.rtvslo.si/arhiv/frekvenca-x/174442156) and for the Slovenian Press Agency (http://znanost.sta.si/2318140/z-zivalskimi-strupi-do-zdravljenja-bolezni-pri-ljudeh).

Figure 2: Science on the Street on animal venoms. Prof. Igor Križaj during presentation of a public lecture entitled "Animal Venoms: from Deadly to the Healthy" in Kavarna Union in Ljubljana. The event, attended by more than 200 people, was organized in the scope of "Science on the Street" project by the Slovenian Academic Society for Science and Engineering (SATENA).

Lipid metabolism and signalization

Tumours display changes in metabolism that maximize their ability to proliferate and survive during times of stress. Apart from the dependence of many cancer types on glucose and glutamine, lipid metabolism is also altered in cancer. An increased availability of fatty acids (FAs), either through *de novo* synthesis in tumour cells or from exogenous sources, is needed for the synthesis of membranes and signalling molecules that are indispensable for tumour growth, and limiting FA supply may prevent cell proliferation in tumours. The transformed properties of cancer cells depend on changes in lipolysis, FA oxidation, membrane phospholipid hydrolysis and reacylation pathways, and the provision of FAs from the circulation. Additionally, accumulation of neutral lipids in cytosolic lipid droplets has been confirmed in several cancers. Discovering the critical links between cancer cell survival and lipid metabolism has a strong therapeutic potential.

Lipid droplets are newly recognized organelles composed of a core of neutral lipids, including triacylglycerol and cholesterol esters, and are covered with a phospholipid monolayer and lipid droplet-associated proteins. They are not passive repositories of energy, but act as platforms integrating cellular lipid metabolism and signalling, FA trafficking, protein management and quality control, viral replication and immunity. However, relatively little is known about the role of lipid accumulation and lipid droplets in cancer. $sPLA_2$s are lipolytic enzymes that hydrolyse membrane phospholipids and release free FAs and lysophospholipids. $sPLA_2$ activity leads to the release of a mixture of mono- and polyunsaturated FAs (PUFAs), including omega-6 and omega-3 PUFAs. We have recently reported a novel mechanism of action of $sPLA_2$ in cancer, describing for the first time a relationship between membrane hydrolysis, lipid droplet formation and breast cancer cell survival. We have found that by inducing lipid droplet formation the $sPLA_2$ enzyme augments cell proliferation and prevents cell death in breast cancer cells exposed to nutrient deprivation-induced stress. Our recent soon to be published data show that lipid droplets help in reducing oxidative stress as well, particularly in cancer cells exposed to starvation or high levels of oxidation-prone PUFAs in the environment. Furthermore, blocking lipid droplets breakdown by depleting cells of a crucial lipase (Figure 3) led to a reduction in PUFA-induced oxidative stress, suggesting that lipid droplets serve as antioxidant storage depots preventing the damage induced by the easily oxidisable PUFAs. Clearly, lipid droplets are emerging as critical regulators of cancer cell survival during stress induced by nutrient deprivation and lipotoxic insults, properties which may be exploited to specifically target cancer cells that are known for their high resilience to oxidative and metabolic stress.

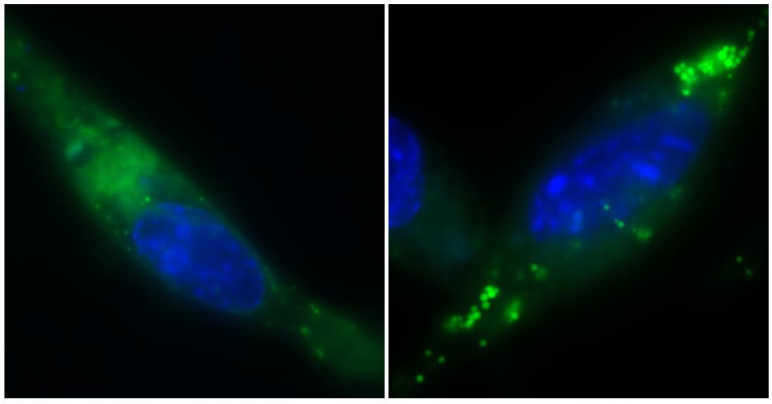

Figure 3: Depleting cells of a lipase involved in lipid droplet breakdown leads to a reduction in lipolysis and a significant increase in the size and number of lipid droplets. Control cells are on the left-hand side and lipase-depleted cells on the right. Lipid droplets were visualized using

neutral-lipid specific staining with the BODIPY 493/503 fluorescence dye and nuclei with DAPI. The photographs were acquired using a Zeiss AxioObserver Z1 microscope.

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

Polygenic trait analysis is one of the fastest developing fields in genetics. Identification of causal alleles for such traits, which cover the vast majority of all, has almost become routine in model organisms like yeast. Application of this knowledge holds great promise for biomedicine and for biotechnology in the development of new cell factories (J.P. Meijnen et al., *Biotechnol. Biofuels*, 9 (2016), 5). Methods for accurate genome editing that enable insertion of causal alleles are necessary for efficient application of such knowledge. To this end, we developed our own protocol of the CRISPR/Cas9 method for genome editing in yeast. This enables us fast and efficient assembly of new combinations of alleles and generation of strains with desired traits. We have been using this approach in the development of yeast strains with varying amounts of neutral lipids.

In 2016 we also made important progress in the field of promoter development for tight regulation of expression of genes – endogenous and heterologous alike – in yeast cells. Promoters are a key element of the synthetic biology toolbox. We therefore optimized methods for accurate determination of the strength and variability of expression of promoter libraries.

A paper was published in 2016 describing the most sophisticated method for large scale microorganism phenotyping (M. Zackrisson et al., *G3*, 6 (2016), 3003–3014). The core of the method, which is based on unprecedently fast and accurate analysis of microorganisms' colony morphology (Figure 4), has been developed at the Jožef Stefan Institute and developed, in collaboration with a group from Sweden, into a key method for polygenic trait analysis.

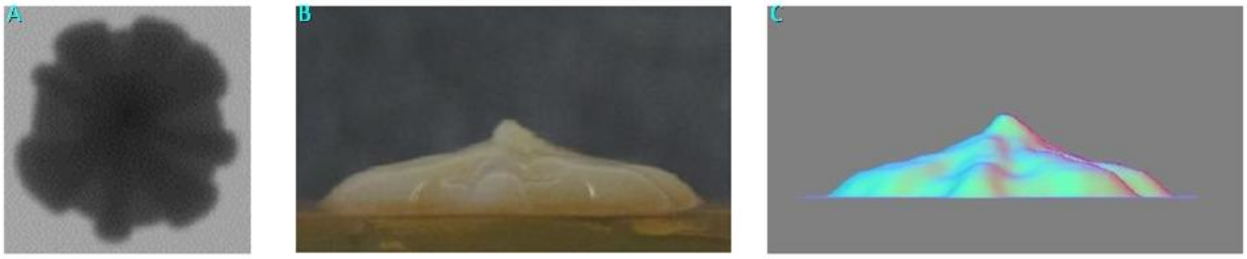

Figure 4: Microorganisms' colony morphology analysis for the Scan-o-matic method. (A) Transmission scanning of a colony. (B) Side view portrait of the colony. (C) Computational reconstruction of the colony's morphology.

Analysis of genomes

S1 family of serine peptidases is the largest family of peptidases. Its members are specifically inhibited by Kunitz/BPTI inhibitors. The Kunitz domain is characterized by a compact 3D structure with the most important inhibitory loops for the inhibition of S1 peptidases. In the present study we analysed the action of site-specific positive selection and its impact on the structurally and functionally important parts of the snake venom Kunitz/BPTI family of proteins (V. Župunski & D. Kordiš**,** *Sci. Rep.*, 6 (2016), 37054). By using numerous models we demonstrated the presence of large numbers of site-specific positively selected sites that can reach between 30–50% of the Kunitz domain. Mapping of the positively selected sites on a 3D model of Kunitz/BPTI inhibitors has shown that these sites are located in the structurally most important part of the molecule, in the inhibitory loops 1 and 2, and also in the Kunitz scaffold

(Figure 5). Amino acid replacements are localized exclusively on the surface, with the vast majority of replacements causing the change of charge. The consequence of these substitutions is the change in electrostatic potential on the surface of Kunitz/BPTI proteins that may play an important role in the precise targeting of these inhibitors into the active site of S1 family of serine peptidases. The presence of the multigene families of Kunitz/BPTIs in venomous snakes can be explained by the target-oriented arms race, since the number of S1 peptidases in vertebrate preys can reach up to hundreds of representatives. As Kunitz/BPTIs are broad spectrum inhibitors, they can be functionally diversified to target numerous and diverse S1 peptidases in their prey. A comparison of the Kunitz/BPTI inhibitors from venomous snakes with those from ticks and vampire bats has demonstrated that they experienced strong and widespread action of site-specific positive selection only in venomous snakes.

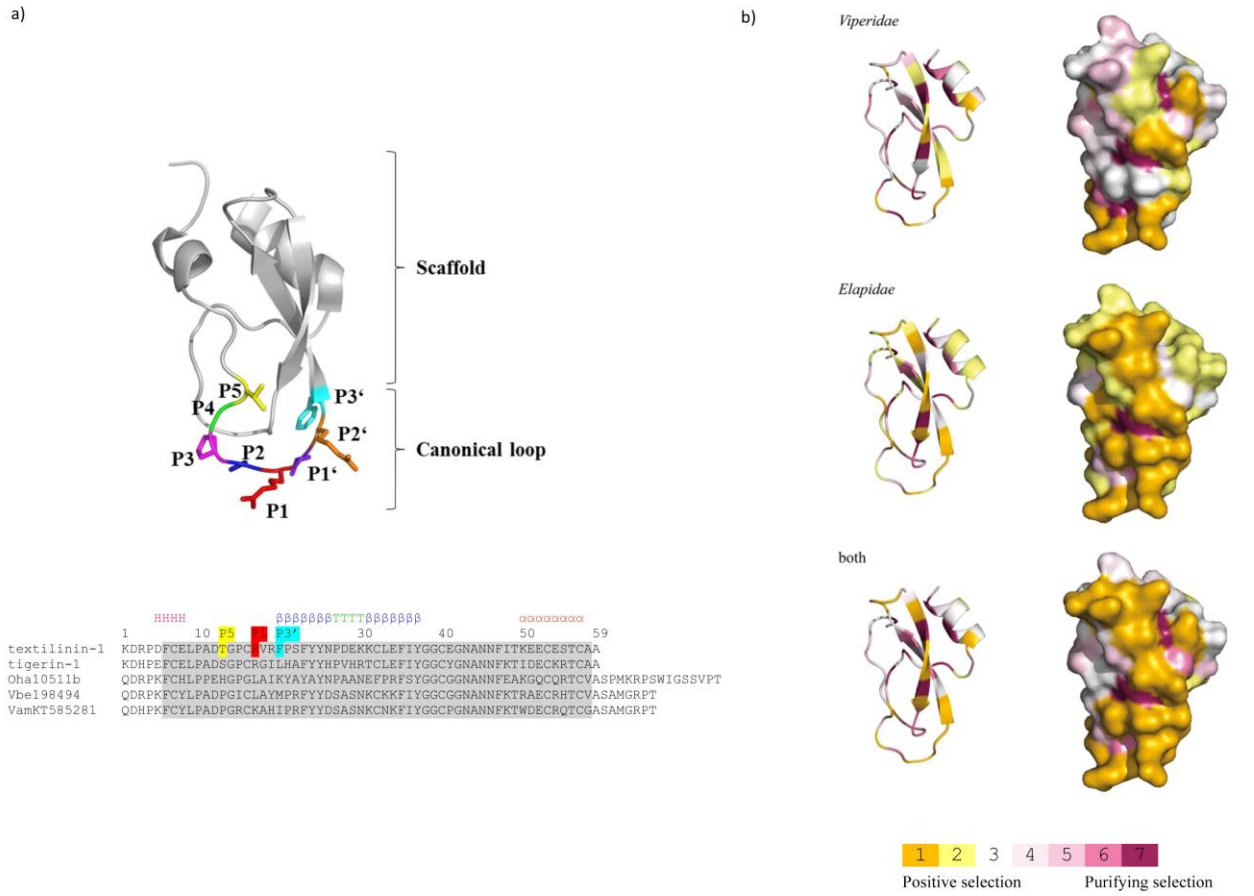

Figure 5: Positively selected amino acids on a three-dimensional model of textilinin-1. a) Structure of textilinin-1 (3BYB) showing the canonical inhibitory loop and protein scaffold. b) Positively selected amino acids in the structure of textilinin-1 (3BYB) are coloured in orange (PP=0.99) and yellow (PP>0.95). The figure is reproduced from V. Župunski & D. Kordiš**,** Sci. Rep*.*, 6 (2016), 37054.

Antimicrobial peptides (AMPs) are one of the oldest defence components of the innate immune system. Although numerous AMP families are present in prokaryotes and eukaryotes, the greatest diversity can be seen in vertebrates. Since AMPs can inhibit the growth of bacteria, fungi and viruses, they are used in the development of novel antibiotics and immuno-regulatory compounds. The evolution of antimicrobial peptides in vertebrates is still not well explained,

mostly because of the confusion in genomic and proteomic databases. A genomic and transcriptomic analysis of all AMP families in vertebrates has been made. In numerous databases we analysed the distribution of AMP families in vertebrates, from the oldest cyclostomes to the land vertebrates. We demonstrated that besides the numerous lineage-specific AMP families vertebrates possess also a number of common AMP families. We have found that the vertebrate ancestor possessed diverse AMP families that survived hundreds of millions years and that evolutionary younger lineage-specific AMP families originated several times independently. We have obtained a new insight into the origin, evolution and functional diversification of numerous AMP families in vertebrates.

Other subjects

In 2016 we also participated at several research projects out of the thematic scope of our department.

Together with our programme group colleagues from the Biotechnical Faculty of the University of Ljubljana (BF UL) we have been analysing the toxin Cdt (cytolethal distending toxin) from the bacteria *Aggregatibacter actinomycetemcomitans* associated with a severe form of periodontal disease. We confirmed the existence of a shorter form of the B subunit of this trimeric toxin (CdtB) on the protein level also in patients with periodontal disease. This finding may result in new insights about this disease and its treatment (D. Obradović et al., *PLoS One*, 11 (2016), e0159231).

With them, we also analysed the influence of OlyA-mCherry, a fluorescent derivative of ostreolysin A from the mushroom *Pleurotus ostreatus*, on Madin-Darby canine kidney cells. We discovered that by binding on cholesterol- and sphingomyelin-enriched membrane nanodomains the derivative induced formation of vesicles into the extracellular space. At detail characterization of the formed vesicles, potentially interesting model for biophysical and biochemical studies of cell membranes and as a system for non-invasive sampling of cytosol from cells, our task was to analyse their protein composition (M. Skočaj et al., *Biochim. Biophys. Acta – Biomembranes*, 1858 (2016), 2882–2893).

We are partners on the targeted research project (CRP) “Definition of molecular parameters for protection of Carniolan honeybee” that is coordinated by the Zootechnical Department at BF UL. In 2016, we continued a comparative proteomic analysis of haemolymph, royal jelly and venom of the local populations of carniolan honeybee (*Apis mellifera carnica*). In this project we also contributed with bioinformatic analysis of the collected data. Our results so far have been preliminarily presented (J. Božič et al., *Acta Agricult. Slov.*, Suppl. 5 (2016), 18–27).

To our colleagues from the Medical Faculty of the UL we successfully assisted with our knowledge of preparation of fluorescently labelled ligands for applications in cell biology. The work describes the internalization of S100B protein from the extracellular space into cultured astrocytes and suggests a new way of removing of this toxic protein also *in vivo* (E. Lasič et al., *J. Neurochem.*, 139 (2016), 309–323).

To our colleagues from the Department of Nanostructured Materials – K7 at the Jožef Stefan Institute we assisted with the mass spectrometric analysis of protein composition of their preparation of fibroin from natural silk, one of the most promising natural materials as a support material in tissue regeneration. The prerequisite for a safe application in a human medicine is namely the preparation of fibroin without a trace of another silk protein sericin due to its high immunogenicity. We succeeded to undoubtedly confirm the perfection of the developed procedure to prepare medically-compatible fibroin from the raw silk (N. Drnovšek et al., *J. Mater. Chem. B*, 4 (2016), 6597–6608).

Researchers from the Department of Physical and Organic Chemistry – K3 at the IJS are also interested in the synthesis of new antimalarial substances. Together with them we prepared a

review paper about malarial toxins in which we suggested several original strategies directed against this group of toxins (K. Starkl Renar et al., Toxicon, 119 (2016), 319–329).

As partners on the project led by colleagues from the Faculty of Electrical Engineering of the 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 publication reporting about the dependency of the protein corona composition of nanoparticles on the procedure of nanoparticles preparation is in press (K. Strojan et al., *PLoS One*).

Most important publications in 2016

1. Župunski, V. and Kordiš, D.: Strong and widespread action of site-specific positive selection in the snake venom Kunitz/BPTI protein family. Sci. Rep., 6 (2016), 37054
2. Latinović, Z., Leonardi, A., Šribar, J., Sajevic, T., Žužek, M.C., Frangež, R., Halassy, B., Trampuš-Bakija, A., Pungerčar, J. and Križaj, I.: Venomics of *Vipera berus berus* to explain differences in pathology elicited by *Vipera ammodytes ammodytes* envenomation: Therapeutic implications. J. Proteomics, 146 (2016), 34–47
3. Kurtović, T., Brvar, M., Grenc, D., Lang Balija, M., Križaj, I. and Halassy, B.: A single dose of Viperfav™ may be inadequate for *Vipera ammodytes* snake bite: A case report and pharmacokinetic evaluation. Toxins, 8 (8) (2016), 244
4. Skočaj, M., Yu, Y., Grundner, M., Resnik, N., Bedina Zavec, A., Leonardi, A., Križaj, I., Guella, G., Maček, P., Erdani-Kreft, M., Frangež, R., Veranič, P. and Sepčić, K.: Characterisation of plasmalemmal shedding of vesicles induced by the cholesterol sphingomyelin binding protein, ostreolysin A-mCherry. Biochim. Biophys. Acta – Biomembranes, 1858 (2016), 2882–2893
5. Obradović, D., Gašperšič, R., Caserman, S., Leonardi, A., Jamnik, M., Podlesek, Z., Seme, K., Anderluh, G., Križaj, I., Maček, P. and Butala, M.: A cytolethal distending toxin variant from *Aggregatibacter actinomycetemcomitans* with an aberrant CdtB that lacks the conserved catalytic histidine 160. PLoS One, 11 (2016), e0159231