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

Secreted phospholipases A_2

One of the major research topics of the department are secreted phospholipases A_2 (sPLA$_2$s) originating from animal venoms that are toxic to humans. We are studying molecular mechanisms of toxic actions of these molecules, particularly their presynaptic neurotoxicity. The knowledge gained working on snake venom sPLA$_2$s is a valuable tool in studying pathological and physiological roles of the mammalian sPLA$_2$ orthologues.

Ammodytoxin A (AtxA) is a presynaptically neurotoxic sPLA$_2$ from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*). In order to get further insight into a role of the phospholipase activity of this molecule in its toxicity we produced a recombinant, enzymatically inactive form of AtxA, AtxA(D49S). This mutant was shown to co-localize with synaptotagmin 1 (Syt1), cytochrome c oxidase subunit II (CcO), protein disulphide isomerase (PDI) and 14-3-3p in PC12 cells, which are used as a model in our laboratory to study cellular dynamics of AtxA. Comparing to the enzymatically active AtxA, the level of co-localization of the enzymatically inactive AtxA was lower with all of the proteins mentioned. In the case of CcO and Syt1, this effect was far more pronounced in non-differentiated (ND) cells compared to differentiated (NGFD) cells, indicating that the phospholipase activity enhances internalization and intracellular trafficking of AtxA to synaptic vesicles and mitochondria to a higher extent in ND cells than in NGFD cells. This suggests that in NGFD cells, the mechanisms leading to the co-localization of both AtxA and AtxA(D49S) with the above mentioned binding proteins, utilize more specific processes than in ND cells. NGFD cells seem to have specific pathways of AtxA internalization and intracellular trafficking that are not dependent on AtxA's enzymatic activity. Additionally, the release of cytochrome c from mitochondria isolated from PC12 cells, caused by AtxA, was determined to be clearly PLA$_2$-activity dependent, since the enzymatically inactive mutant AtxA(D49S) was unable to elicit this effect.

AtxA binds PDI in the lumen of the endoplasmic reticulum (ER) of PC12 cells also *in vivo*. In a recently published paper we suggested that this interaction is important for the translocation of AtxA from the extracellular space into the cytosol of the cell (J. Oberčkal et al., *PLoS One*, 10 (2015), e0120692). Besides assisting AtxA to migrate retrogradely from the Golgi apparatus to the ER, PDI can also help AtxA to translocate across the ER membrane. The results reported here also strongly support the hypothesis that PDI partakes at the retrograde cellular transport of mammalian sPLA$_2$s, structurally related to AtxA. The sPLA$_2$–PDI model that we present provides a detailed structural insight into the interaction between these proteins (Figure 1), enabling thus a targeted study of the sPLA$_2$ cell internalization process.

Springer publishing company invited us to prepare a chapter for their monograph Snake Venoms, which is currently in press (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 are invitations to highly attended Toxicology

2015 meeting organized by the Slovenian Medical Society in Ljubljana (I. Križaj) and to the World Congress of the International Society on Toxinology—IST in Oxford (I. Križaj).

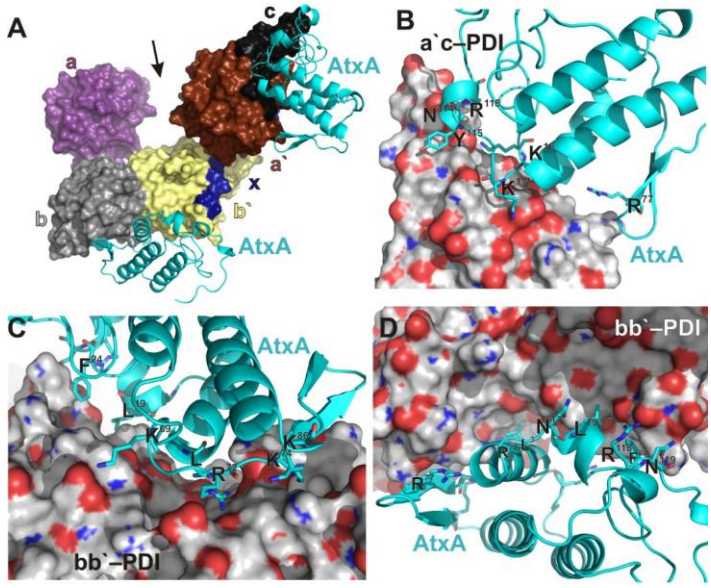

Figure 1: Three-dimensional model of the complex between AtxA and PDI. (A) Molecular modelling resulted in two solutions for AtxA binding to PDI. Both solutions are displayed on the same molecule of PDI. Arrow points towards the active side of PDI. According to the first solution (B), AtxA binds to PDI between domains a' and c of PDI (a'c-model or binding site), while according to the second solution (C and D), AtxA binds at domains b and b' (bb'-model or binding site). In the case of human PDI only the bb'-model is possible.

Other pharmacologically active components from natural venoms

In 2015 we continued with the systematic analysis of the venom components (venom proteomics or venomics) of the nose-horned viper (*Vipera ammodytes ammodytes*) and the common adder (*Vipera berus berus*) venoms. We have been analysing proteomic data and complemented them with the transcriptomic data obtained by analysis of the nose-horned viper venom gland cDNA library. Two publications are in preparation.

With intensity we studied the snake venom proteins that affect the blood coagulation process—haemostasis. In this area of research, we succeeded to publish a structural and biochemical description of a monomeric alfa-fibrinogenolytic metalloproteinase, VaF1 (A. Leonardi et al., *Biochimie* 109 (2015), 78–87). VaF1 would be expected to exert anticoagulant action due to its hydrolysis of fibrinogen, factor X, prothrombin and plasminogen, *i.e.*, plasma proteins involved in blood coagulation. In standard experimental conditions, VaF1 was not recognised by antiserum against the whole venom, therefore, it tentatively contributes to post-serotherapy complications, such as ineffective blood coagulation, in the envenomed patient.

In 2015, two additional interesting groups of molecules from the venom of the nose-horned viper attracted our attention, namely disintegrins and CRISPs (Cysteine RIch Secretory Proteins). Disintegrins are polypeptides that bind to integrin molecules and impair in this way their function. This subject was a research topic of a student team from the Jurij Vega High School in Idrija that worked at the department under our supervision. Our results demonstrated that these molecules efficiently prevent migration and thus spreading of cancer cells. This confirms their anti-metastatic potential and gives a good prospective for their development in the direction of a new anti-cancer drug. The high school research work has been awarded by the prestigious Krka Award (Figure 2) and achieved also a resounding international success by winning the first place at the South America's largest international natural sciences and technology contest Mostratec in Brazil.

CRISPs are toxic and they block ion channels in different cells, causing, for example, the paralysis of peripheral smooth muscles and hypothermia. In this year we looked for the physiological effects of CRISPs isolated from the nose-horned viper venom in different experimental settings in collaboration with our colleagues at the Veterinary Faculty, University of

Ljubljana (UL) and the Strathclyde University in Glasgow (Scotland, UK). Unfortunately, we still do not have a clear interpretation of the action of these proteins.

Figure 2: From the 45th Krka Award ceremony for the best high school research achievements. The awarded team of students from the Jurij Vega High School in Idrija with their tutor from our department Dr. Adrijana Leonardi and the High School Headmaster. Dr. Toni Petan, the co-tutor from the Jožef Stefan Institute, did not attend the ceremony. Source: Archive of Krka pharmaceutical company, Novo mesto.

Lipid metabolism and signalization

Changes in lipid metabolism in cancer are novel therapeutic targets

Dysregulated lipid metabolism is a fundamental metabolic alteration that enables cancer cell survival and sustains rapid growth and proliferation. 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. Discovering the weakest points in the core of these metabolic requirements in lipid metabolism has thus a strong therapeutic potential.

Mammalian $sPLA_2$s are lipolytic enzymes that hydrolyse membrane phospholipids to liberate free FAs and lysophospholipids. The group X $sPLA_2$ ($sPLA_2$-X) is the most potent mammalian $sPLA_2$ in hydrolysing cell membranes and its activity leads to the release of a mixture of mono- and polyunsaturated FAs (PUFAs), including omega-6 and omega-3 PUFAs, which have many downstream signalling and metabolic effects. We have recently reported a novel mechanism of action of $sPLA_2$-X in cancer, describing for the first time a relationship between membrane hydrolysis, changes in lipid accumulation and breast cancer cell survival. We have found that $sPLA_2$-X induces lipid droplet (LD) formation (Figure 3), stimulates FA oxidation and suppresses lipogenesis. These metabolic changes in turn augment cell proliferation and prevent cell death during metabolic stress. LDs are newly recognized organelles composed of a core of neutral lipids, including triacylglycerol (TAG) and cholesterol esters, and are covered with a phospholipid monolayer and LD-associated proteins. They are not passive repositories of energy, but act as platforms integrating cell signalling and metabolism, and elevated amounts of LDs have been

discovered in tumour tissue of cancer patients. Our recent unpublished data show that, besides breast cancer, $sPLA_2$-X induces LD formation in other cancer cells *in vitro*, such as colorectal, ovarian, endometrial, neuroblastoma, leukemia and cervical cancer cells. This suggests that its effects on lipid metabolism are not restricted to breast cancer cells. We have also found that exogenous addition of PUFAs to cancer cells mimics the effect of $sPLA_2$-X on lipid accumulation, but in contrast to oleic acid and $sPLA_2$-X, they are toxic to highly invasive cells. Currently, we are using quantitative PCR and proteomic analyses to identify changes in the amount and composition of LD-associated proteins in breast cancer cells with high amounts of $sPLA_2$-induced LDs. We have also identified two inhibitors with therapeutic potential for the simultaneous reduction of LD accumulation and cancer cell survival.

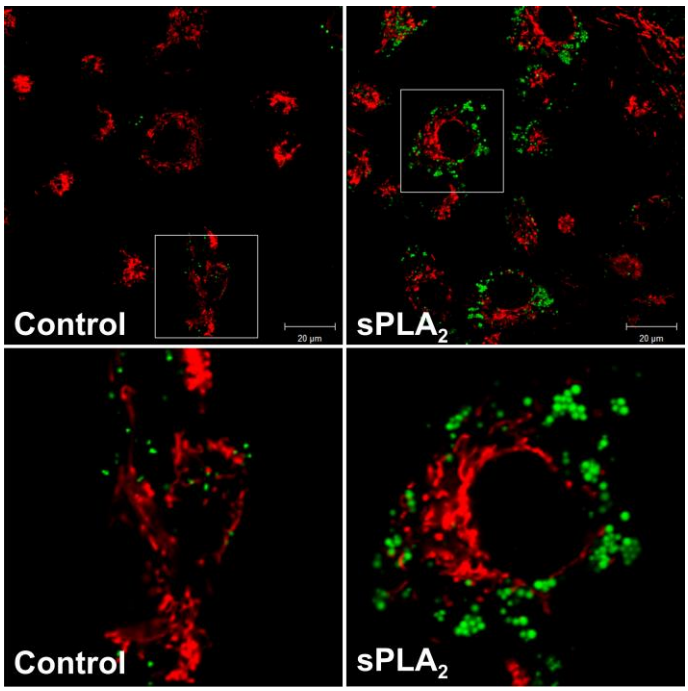

Figure 3: The human $sPLA_2$-X enzyme induces formation of lipid droplets in breast cancer cells. Control cells are on the left-hand side and $sPLA_2$-treated cells on the right. Mitochondria were stained with Mitotracker Red (red signal), while lipid droplets were visualized using neutral-lipid specific staining with the BODIPY 493/503 fluorescent dye. The photographs were acquired using a Zeiss LSM 710 confocal microscope.

Activity of our group in the field of $sPLA_2$ is evidently well known also to the editors of the *Digestive Diseases and Sciences* as they invited us to write an editorial about the role of enzyme $iPLA_2\beta$ in the maintenance of intestinal homeostasis and its possible association with occurrence of the ulcerative colitis (T. Petan and I. Križaj, *Dig. Dis. Sci.*, 60 (2015), 3504–3506).

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

Recent development of genomics enabled transfer of the polygenic trait analysis methods into biotechnological applications of new cell factories design. In 2015 we continued coordinating a European consortium whose aim is to combine these methods with metabolic engineering and synthetic biology tools to develop new generation cell factories (M. Kavšček et al., *Microb. Cell*

Fact., 14 (2015), 94). Such cell factories will play an important role in the development of bioeconomy for the production of biofuels (U. Petrovič, *Yeast*, 32 (2015), 583–593) and other compounds that are currently made from non-sustainable feedstocks.

In the field of inter-organelle communication, currently one of the hottest topics within cell biology, we demonstrated a new way of interaction between mitochondria and peroxisomes (Figure 4), based on the interaction between ERMES complex and peroxisomal protein Pex11 (M. Mattiazzi Ušaj et al., *J. Mol. Biol*., 427 (2015), 2072–2087). The paper attracted a lot of attention and paved the way for some additional interpretations of the organization of eukaryotic cells that until now were considered non-conventional.

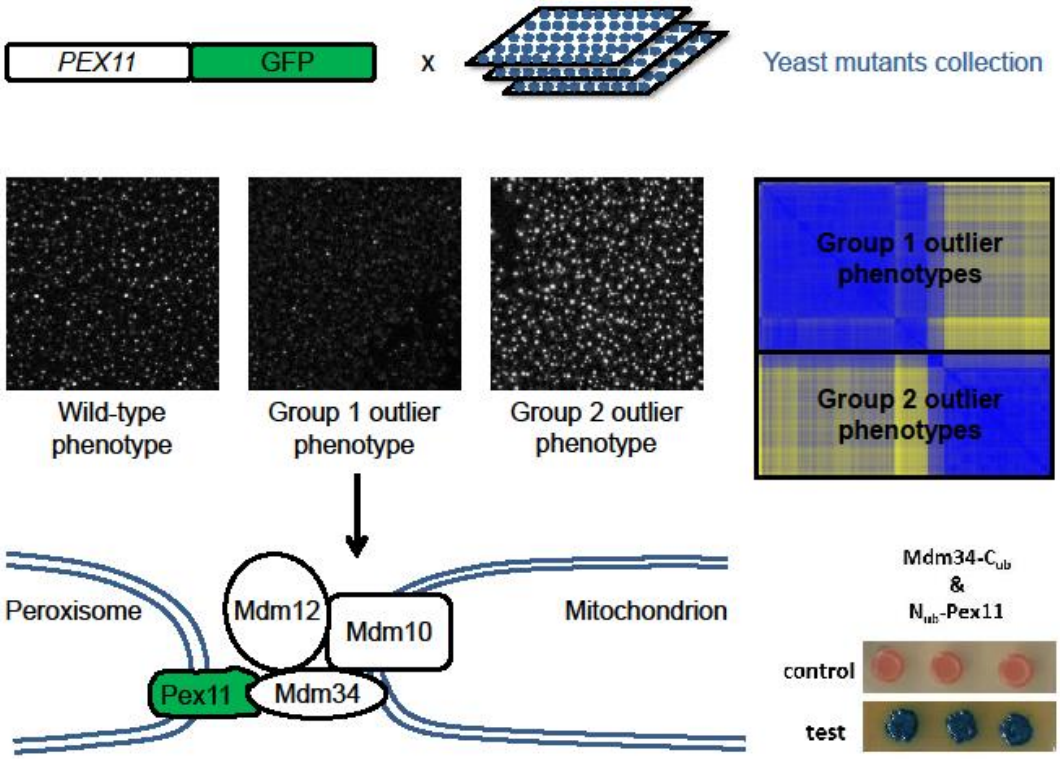

Figure 4: Genome-wide study was performed to analyse the localization of Pex11, a peroxisomal protein. Bioinformatics algorithms were used to predict the molecular basis for the observed phenotypes and a corresponding model. Validity of the model of the interaction between Pex11 and ERMES complex components was confirmed with follow-up experiments.

We also published the results of the longer-term study on the physiological interconnection between zinc and yeast adiponectin receptor homolog (M. Mattiazzi Ušaj et al., *Metallomics*, 7 (2015), 1338–1351). It is expected that the results of this study on the model organism will spur new ideas for the control or treatment of metabolic syndrome and type 2 diabetes, two of the currently most severe medical problems of our civilization in which the adiponectin receptor plays an important role.

Analysis of genomes

Evolutionary genomics

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 (2015), ISBN: 78-94-007-6648-8) we highlighted how the progress in the last decade has increased our understanding of the evolution of $sPLA_2$ toxins in venomous animals (Figure 5).

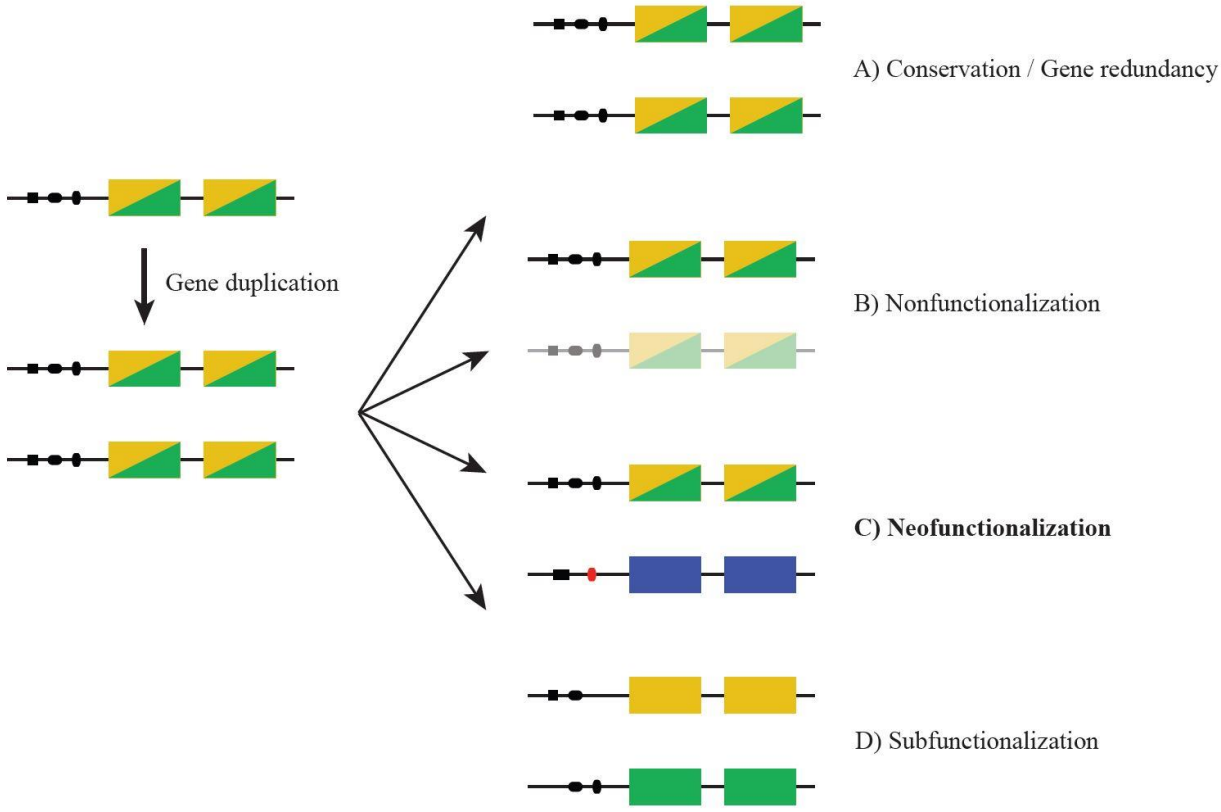

Figure 5: Evolutionary fates of gene copies after duplication. On the left side, schematic representation of a gene undergoing gene duplication (exons are depicted as yellow/green blocks and regulatory elements as geometric forms). On the right side, the four major possible evolutionary fates of the copies are represented: (a) both copies remain essentially unchanged and functionally redundant (conservation/gene redundancy). (b) One of the gene copies is deleted from the genome (or pseudogenized), restoring the initial situation (nonfuntionalization). (c) One of the copies accumulates mutations in its coding sequence and/or associated regulatory elements (in red and blue), thereby acquiring new gene functions (neofunctionalization). (d) Coding sequences and regulatory elements may also evolve and be partitioned according to the specific roles played by each subfunctionalized gene copy (exons indicated as yellow or green only) (subfunctionalization).

RNA viruses are common pathogens of human, animals and economically important plants, which is why they have a big influence on economy, medicine, agriculture and technology. Numerous studies have been conducted about the RNA viruses in insects since they represent major vectors for transmission of these viruses. The knowledge about RNA viruses in butterflies and moths (Lepidoptera) was until now quite limited. In the lepidopteran transcriptomes we found 12 novel families of RNA viruses. We found two new families of double stranded RNA viruses (*Partitiviridae* and *Totiviridae*), seven new families of single stranded RNA(+) viruses (*Betaflexiviridae*, *Dicistroviridae*, *Narnaviridae*, *Negevirus*, *Potyviridae*, *Tombusviridae* and *Virgaviridae*) and three new families of single stranded RNA(-) viruses (*Bunyaviridae*, *Nyamiviridae* and *Orthomyxoviridae*). Only single stranded RNA viruses (*Nyamiviridae*, *Orthomyxoviridae*, *Rhabdoviridae*, *Negevirus*, *Virgaviridae*) have been found endogenised in the lepidopteran genomes. We found that lepidopterans can transmit five families of plant-specific RNA viruses. Analysis of transcriptomes and genomes has shown that butterflies contain numerous novel families of RNA viruses.

In the field of cysteine proteases and their inhibitors (cystatins) we studied the origin and evolution of 11 orthologous gene families that are present in evolutionary older lineages of vertebrates. Cystatin superfamily contain 20 orthologous families in vertebrates and we analysed the following orthologous gene families: cystatin C, cystatin F, cystatin E/M, latexin, TIG1, cathelicidin, Spp24, fetuin A, fetuin B, HRG and kininogen. Functional diversification of the cystatin superfamily in vertebrates was connected to the loss of their inhibitory activity and the gain of novel biological roles. To explain the functional diversification of the cystatin superfamily, we clarified the origin and evolution of the above mentioned orthologous gene families. We found that cystatin superfamily was involved in several “vertebrate innovations”, such as skeletogenesis and adaptive immune system, and important roles were gained in innate immunity and reproduction. Novel vertebrate cathepsins also adopted important roles in adaptive immune system and skeletogenesis. We explained the co-evolution of orthologous gene families of the cystatin superfamily with their interaction partners (cathepsins and some novel proteins) and their involvement in the newly gained systems of vertebrates.

Cysteine peptidases that belong to the family C1A peptidases are one of the largest groups among peptidases. Their biological roles are much better known in eukaryotes than in prokaryotes. Peptidases in prokaryotes can participate in the process of pathogenesis, but the mechanisms are still quite unknown. The protein domains that are associated with the peptidases can have a role of virulence factors. The availability of a huge number of prokaryotic genomes and proteomes has allowed us to analyse the diversity of the C1A peptidases in prokaryotes. We investigated the distribution and domain architectures in C1A papain superfamily in prokaryotes. Distribution of C1A peptidases in prokaryotes is rather limited, which explains their intended specific function. The number of multidomain C1A proteins in prokaryotes is much greater than one would expect given their distribution. Even their domain architectures are extremely structurally diverse. The connections of multidomain C1A peptidases with the exceptionally large number of different protein domains have confirmed that these domains are relevant to microbial adaptations for survival in the host. The analysis of the structural diversity of the C1A peptidases in prokaryotes has provided a new insight into their biological roles that are not limited only to the pathogenesis.

Other subjects

In 2014 we also participated at several research projects out of the thematic scope of our department.

With structural identification we collaborated with our colleagues from the Department of Biology, the Biotechnical Faculty of UL (BF UL), on comparative composition analysis of protein

corona of three different carbon-based nanoparticles after their exposure to human serum (M. Sopotnik et al., *Carbon*, 95 (2015), 560–572). Similar analysis, just with nanoparticles of different structure, we conveyed in collaboration with the team from the Faculty of Electrical Engineering of UL. In this study we related protein corona composition of nanoparticles and their physical characteristics with the mode of preparation of these nanoparticles, meaning steps between their synthesis and application in one of the biologically relevant systems.

In collaboration with another group from the Department of Biology at BF UL, we analysed the toxin Cdt (cytolethal distending toxin) from 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.

In consortium, coordinated by colleagues from the Institute of Biochemistry, the Medical Faculty of UL (MF UL), we participated at similarity-based virtual screening and synthesis of new benzoic acid-derived antifungal compounds directed against cytochrome P450 monooxygenase CYP53 enzymes of animal and plant pathogenic fungi (S. Berne et al., *Bioorg. Medic. Chem.*, 23 (2015), 4264–4276).

In collaboration with another group from the MF UL we successfully applied our $sPLA_2$ expertize. In this study we discovered that the patients suffering from ovarian endometriosis have elevated expression of the group IIA $sPLA_2$ (PLA2G2A) on both the mRNA and protein levels. Our data indicate that PLA2G2A is implicated in the pathophysiology of ovarian endometriosis, but that it cannot be used as a diagnostic biomarker (V. Kocbek et al., *Gynecol. Endocrinol.*, 31 (2015), 214–218).

By performing surface plasmon resonance (SPR) measurements, we collaborated 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 (M. Faridounnia et al., *J. Biol. Chem.*, 290 (2015), 20541–20555).

With structural analyses we participated at optimization of expression of mouse perforin in insect cells and purification of the recombinant protein in the project led by colleagues from the National Institute for Chemistry in Ljubljana (O. Naneh et al., *J. Immunol. Methods*, 426 (2015), 19–28).

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 2015, we accomplished comparative proteomic analysis of haemolymph, royal jelly and venom of the local populations of carniolan honeybee (*Apis mellifera carnica*). We also contributed at bioinformatic analysis of the collected data.

Most important publications in 2015

1. Oberčkal, J., Kovačič, L., Šribar, J., Leonardi, A., Dolinar, K., Pucer Janež, A. and Križaj, I.: On the role of protein disulphide isomerase in the retrograde cell transport of secreted phospholipases A_2. PLoS One, 10 (2015), e0120692
2. Leonardi, A., Sajevic, T., Latinović, Z., Pungerčar, J., Lang Balija, M., Trampuš Bakija, A., Vidmar, R., Halassy, B. and Križaj, I.: Structural and biochemical characterisation of VaF1, a P-IIIa fibrinogenolytic metalloproteinase from *Vipera ammodytes ammodytes* venom. Biochimie, 109 (2015), 78–87
3. Mattiazzi Ušaj, M., Prelec, M., Brložnik, M., Primo, C., Curk, T., Ščančar, J., Yenush, L. and Petrovič, U.: Yeast *Saccharomyces cerevisiae* adiponectin receptor homolog Izh2 is involved in the regulation of zinc, phospholipid and pH homeostasis. Metallomics, 7 (2015), 1338–1351
4. Mattiazzi Ušaj, M., Brložnik, M., Kaferle, P., Žitnik, M., Wolinski, H., Leitner, F., Kohlwein, S.D., Zupan B. and Petrovič, U.: Genome-wide localization study of yeast Pex11 identifies

peroxisome-mitochondria interactions through the ERMES complex. J. Mol. Biol., 427 (2015), 2072–2087
5. Petrovič, U.: Next generation biofuels: a new challenge for yeast. Yeast, 32 (2015), 583–593