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.

Secreted phospholipases A_2

One of the major research topics of the department are secreted phospholipases A_2 ($sPLA_2$s) originating from animal toxins as well as those found in humans. We are studying the molecular mechanisms of action of the toxic $sPLA_2$s, particularly those endowed with presynaptic neurotoxicity, and the role of endogenous $sPLA_2$s in pathological and physiological processes in mammals.

Along with the development of a new way of renaturation of recombinantly expressed $sPLA_2$s we have endeavoured to produce an enzymatically inactive form of ammodytoxin A (AtxA), a neurotoxic $sPLA_2$ from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*). We succeeded in this by replacing Asp with Ser in the active site of AtxA. Analysis with mass spectrometry and the binding properties of the mutant AtxA(D49S) showed that the protein is properly folded. We expect that the use of this molecule will lead to progress in the understanding of the role of phospholipase activity in the neurotoxicity of $sPLA_2$s, as well as the mechanism of $sPLA_2$ transport across the plasma membrane and between different cellular compartments. We have already prepared a fluorescently labelled derivative of the AtxA(D49S) mutant and performed preliminary studies on the rat PC12 cell line, which is a model used in our laboratory to study the dynamics of AtxA cellular uptake, its co-localization with mitochondria and certain intracellular proteins with the use of confocal microscopy. Additionally, we have shown that AtxA binds protein disulphide isomerase in the lumen of the endoplasmic reticulum of PC12 cells also *in vivo* (Figure 1).

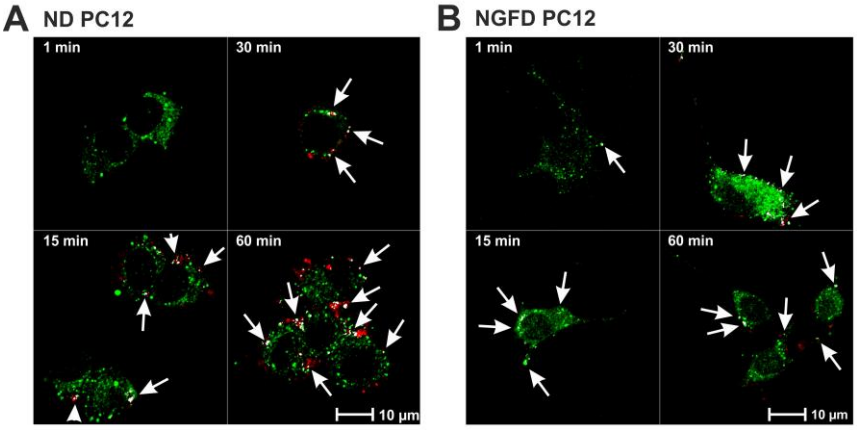

Figure 1: Atx and PDI co-localize in living PC12 cells. (A) Non-differentiated (ND) and (B) NGF-differentiated (NGFD) PC12 cells were incubated in the presence of 100 nM ^{546}Alexa-Atx (red signal) for indicated times. Cells were fixed and protein disulphide isomerase (PDI) stained with anti-PDI antibodies (green signal). Co-localized green and red pixels are shown in white. Arrows point to most extensive areas of co-localization.

In a recently prepared paper we suggest 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*, *PLoS One*, 10(3) (2015), e0120692). Calmodulin (CaM) is a regulatory protein in the cell cytosol, presumably very important for the intracellular action of AtxA and its mammalian $sPLA_2$ homologues. In order to dynamically monitor the interaction of AtxA and CaM in mammalian cells using the FRET method, we have continued with the development of fluorescent derivatives of both proteins.

We set out to identify the $sPLA_2$ receptor in the presynaptic membrane of motoneurons (N-$sPLA_2$R), which plays a crucial role in the neurotoxic effect of these molecules, by using OS_2, an AtxA-like $sPLA_2$ from the venom of the coastal taipan (*Oxyuranus scutellatus scutellatus*) since it binds to N-$sPLA_2$R with a more than 1000-fold higher affinity than AtxA. In collaboration with the research group from Institut de Pharmacologie Moléculaire et Cellulaire, Centre national de la recherche scientifique (CNRS) in Valbonne, France, we continued with the characterization of the photo-reactive derivatives of the recombinant wild type OS_2 and its chimera with the non-toxic OS_1 from the venom of the same snake, which we will use for the identification of the N-$sPLA_2$R.

$sPLA_2$s are a physiologically very important family of multifunctional proteins. Their effects do not always depend on their enzymatic activity but in certain cases also on their binding to other molecules. To discover new $sPLA_2$ binding molecules we used immunoaffinity chromatography. In the venom of the nose-horned viper we have identified an Atx-binding protein that inhibits the activity of chymotrypsin and belongs to the Kunitz-type protease inhibitors (ChI). Our first results show that the toxicity of AtxA is higher in the presence of ChI. ChI-like molecules can also be found in mammals. It will be interesting to check their affinity to $sPLA_2$s and determine the physiological effects of their interaction with $sPLA_2$s (M. Brgles et al., *Anal. Bioanal. Chem.*, 406 (2014), 293–304).

In an attempt to prepare an effective antiserum against the nose-horned viper venom we have discovered that the content of Atx in the venom is in a positive correlation with the level of its immunogenicity. A fast and accurate method for the determination of Atx content in the venom is therefore one of the crucial steps to develop an effective procedure for the production of a quality antiserum. Together with the colleagues from the Technische Universität Vienna, Austria, we have made a step further in the Atx content characterization in the venom by developing the original method, enabling the purification of all three very similar forms of this toxin in only one step (V. U. Weiss et al., *Electrophoresis*, 35 (2014), 2137–2145).

We were invited to submit a review paper to the *Toxicon* journal (J. Šribar et al., *Toxicon*, 89 (2014), 9–16). We presented a critical overview of all the important research on the action of presynaptically neurotoxic $sPLA_2$s reported since our last review in 2007, proposed a hypothesis about the mechanism of action of these toxins and suggested further approaches to test it.

Previous studies have shown that altered expression of different groups of $sPLA_2$s is related to pathological changes of the different types of cancer, such as cancer of the colon and rectum, stomach, esophagus, ovary and prostate. In this respect, it is most likely that either the pro-tumorigenic or anti-tumorigenic role of a certain group of $sPLA_2$s depends on the particular type of cancer. Despite the fact that the connection of some mammalian $sPLA_2$s with different types of cancer has been recently confirmed, their role in breast cancer is still poorly elucidated. In 2014, we published the results of our in-depth studies where we have found that the mRNA expression of $sPLA_2$ groups IIA, III, and X is different both *in vivo*, in tumor biopsies, and *in vitro*, in various cancer cells, from that of the non-cancer cells (V. Brglez et al., *Biochem. Biophys. Res. Commun.*, 445 (2014), 230–235). We have found that one of the main causes for the observed differences in the expression of $sPLA_2$s is modified epigenetic regulation of their gene expression in breast cancer cells. Expression of $sPLA_2$s is thus differentially regulated by DNA methylation and histone acetylation, where appropriate (*i.e.*, their) genes are most highly silenced in the aggressive, triple-negative breast cancer cells, MDA-MB-231, due to the

combined action of both mechanisms. The transcription start site promoter region and the upstream CpG islands (Figure 2), exclusive to the group X $sPLA_2$ gene, have variable roles in the regulation of $sPLA_2$ expression.

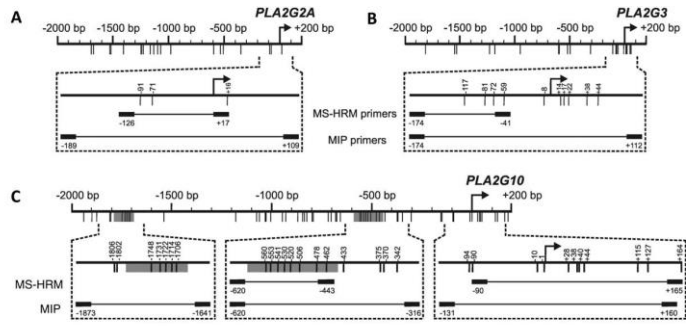

Figure 2: Schematic representation of the promoter gene regions of human group IIA (*PLA2G2A*), III (*PLA2G3*) and X (*PLA2G10*) $sPLA_2$. The promoter region of each $sPLA_2$ gene was analysed in an area from 2000 base pairs upstream to 200 base pairs downstream of the putative transcription start site (TSS, indicated by an arrow), revealing two CpG islands (denoted by gray boxes; individual CG doublets are marked by vertical dashes) in the *PLA2G10* promoter at the positions around –1700 and –500 relative to the TSS, and none in the promoters of *PLA2G2A* and *PLA2G3*. The regions that were analysed by two different methods, MIP ("methylation-independent polymerase chain reaction") and MS-HRM ("methylation-specific high resolution melting"), and using sequence-specific oligonucleotide primers are shown enlarged. The figure is reproduced from V. Brglez et al., *Biochem. Biophys. Res. Commun.*, 445 (2014), 230–235.

Recently, in 2013, we also found that exogenously added human $sPLA_2$-X, but not $sPLA_2$-IIA, induced lipid droplet formation, particularly in the highly tumorigenic MDA-MB-231 cells in an enzymatic activity-dependent manner, thereby stimulating cell proliferation and significantly prolonging cell survival under serum (nutrient) deprivation-induced stress. The results also suggested that free fatty acids, in particular oleic acid, released from membrane phospholipids by the enzymatic action of $sPLA_2$-X, are primarily responsible for these effects. In accordance with these findings, the pro-tumorigenic effect of $sPLA_2$-X was particularly evident in the increased survival (viability) of highly tumorigenic cells, but not of that of non-tumorigenic and weakly tumorigenic breast cancer cells. Our latest research, published in 2014, thus convincingly show that the differential expression of human $sPLA_2$-IIA, -III and -X in breast cancer cells is due to the different level of epigenetic silencing, both by hypermethylation of genomic DNA and deacetylation of histone proteins, of particular phospholipase genes.

Together with the Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, we participated in a study on the role of human $sPLA_2$-IIA in ovarian endometriosis. The latter is a heterogeneous progressive disease, with the presence of endometrioma (cysts) in the ovaries. It is a leading cause of chronic pelvic pain and subfertility, and it can affect up to ten percent of women of reproductive age. The availability of less invasive and timely diagnostic methods for women with endometriosis would thus be highly beneficial. Previous research of the colleagues from the Faculty of Medicine has also indicated that $sPLA_2$-IIA could serve as a potential biomarker for ovarian endometriosis. One-hundred and sixteen women, 70 among them with ovarian endometriosis, were included in the investigation that was published online last year (V. Kocbek et al., *Gynecol. Endocrinol.*, in press). In the cases of ovarian endometriosis, we observed a significant increase in the synthesis of $sPLA_2$-IIA mRNA in cancer tissue (endometrioma) compared with normal endometrium, but not the higher concentration of the $sPLA_2$-IIA protein in the peritoneal fluid and serum. The results obtained show that $sPLA_2$-IIA is involved in pathophysiological changes in the ovarian endometriosis, but it is not useful as a diagnostic biomarker for this disease.

Based on our renowned research of the (patho)physiological role of $sPLA_2$s, we have been invited to prepare a review on the different operating modes $sPLA_2$s in cancer, which was published in the last year (V. Brglez et al., *Biochimie*, 107 (2014), 114–123). Mostly it reflects the action of already mentioned three groups of $sPLA_2$s, whose altered expression has been observed in several cancer types both in mouse models and in patients, namely IIA, III and X. It is becoming increasingly clear that the involvement of $sPLA_2$s in various types of cancer includes several modes of operation of these membrane-active phospholipase enzymes, depending on a particular cell environment. Firstly, already some time ago, the role of $sPLA_2$s in cancer has been connected with their enzyme activity and the consequent release of free fatty acids, *i.e.*, basic building blocks in the synthesis of an array of biologically active lipid mediators, particularly the products of arachidonic acid, called eicosanoids that promote tumorigenesis by inhibiting cell death (apoptosis), increasing local inflammation and formation of new blood vessels (angiogenesis). Secondly, many of the biological effects are independent of the enzyme (phospholipase) activity and indicate an additional role of receptors in the action of $sPLA_2$s in the development of cancer. And thirdly, the latest research, of which a large proportion was carried out by our group, discovered a very important role of $sPLA_2$s in the regulation of basic lipid metabolism (lipid droplet formation and lipid degradation in the mitochondria), which may affect various physiological and pathological cellular changes, including the formation and development of cancer.

Activity of our group in the field of $sPLA_2$ is evidently well known also to the editors of the *Protein and Peptide Letters* as they invited us to prepare a review article on the role of these molecules in the mammalian immune system (I. Križaj, *Protein Pept. Lett.*, 21 (2014), 1201–1208).

Other pharmacologically active components from natural toxins

In 2014 we continued to systematically analyse the components of the nose-horned venom that affect the blood coagulation process – haemostasis (Figure 3). We succeeded to publish a description of heterodimeric haemorrhagic metalloproteinase, VaH4 (A. Leonardi et al., *Toxicon*, 77 (2014), 141–155).

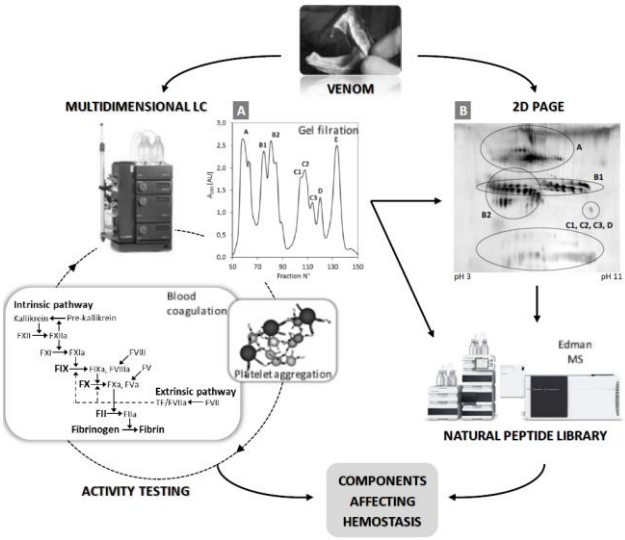

Figure 3: Proteomic analysis of haemostaticly active components in the nose-horned viper venom. (A) Initial fractionation of the venom proceeded on Sephacryl S-200. (B) 2D PAGE analysis of the crude venom. Groups of proteins found in particular size-exclusion chromatography fraction (A) are encircled. The figure is reproduced from T. Sajevic et al., *Toxin Rev.*, 33 (2014), 33–36.

Upon editor's invitation we prepared a review paper describing our research on haemostatically active components from the venom of the nose-horned viper (T. Sajevic et al., *Toxin Rev.*, 33 (2014), 33–36). With our colleagues from the Imunološki zavod Zagreb, Croatia, and Technische Universität Vienna, Austria, we described another very interesting molecule from the venom of this snake. The serine proteinase VaSP1 with an unconventional structure of the active site, according to its substrate specificity and the fact that it prolongs the prothrombin and the activated partial thromboplastin time, very likely possesses an anticoagulant effect (T. Kurtović et al., *Toxicon*, 77 (2014), 93–104).

In 2014, two additional interesting groups of molecules from the venom of the nose-horned viper were a focus of our research, namely disintegrins and CRISPs (Cysteine RIch Secretory Proteins). Disintegrins are polypeptides that bind to integrin molecules and thus impair their function. CRISPs are toxic and they block ion channels in different cells, causing, for example, the paralysis of peripheral smooth muscles and hypothermia. CRISPs and disintegrins from the venom of the nose-horned viper were purified and biochemically characterized, while the research on their pathophysiological effects is still being conducted.

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

In the field of genetics, in the last years we have seen a rapid development of techniques and methods for polygenic traits analysis, which have been spurred by recent developments in genomics. Our group started coordinating a European consortium whose aim is to combine these methods with metabolic engineering and synthetic biology tools, in order to develop complex cell factories that in the future will play a very important role in the development of bioeconomy (https://krog.sta.si/2079947).

Inter-organelle communication is a rapidly developing subfield of cell biology that plays a crucial role for system-wide understanding of cells. In 2014 we finalized a study in which we discovered a new way of interaction between mitochondria and peroxisomes. This discovery could have an important impact on the design of biotechnological processes for lipid-based bioproducts production, and on the understanding of the development of metabolic syndrome in humans.

Our colleague from the department, currently a postdoc at the University of Toronto, participated in the development of a new method to analyse protein-protein interactions, the mammalian-membrane two-hybrid assay (J. Petschnigg et al., *Nat. Methods*, 11 (2014), 585–592).

In 2014 we took over the organization of ISSY31 conference – International Specialised Symposium on Yeast, which gathered 250 world-leading researchers from the field of yeast fermentations (Figure 4). We also co-organized an international workshop on functional genomics (http://biolab.github.io/functional-genomics-workshop/), which attracted over 50 PhD students and post-doctoral fellows from Slovenia and abroad.

Neonicotinoid insecticides were rather notorious in 2013 because of their proposed toxicity for bees and other non-target organisms, on the basis of which they were banned in the EU in April 2013. Using chemogenomics analysis in the yeast model we have determined side effects of neonicotinoid insecticides, and especially of additives from insecticide formulations. We have shown examples where additives are even more toxic than neonicotinoids themselves (M. Mattiazzi Ušaj et al., *Chemosphere*, 104 (2014), 91–96).

Figure 4: 31st International Specialised Symposium on Yeast, covering all the aspects of yeast fermentations, took place in the Vipava valley in October 2014. The conference was co-organized by our department with Prof. Uroš Petrovič as the executive chairman of the organizing committee.

Pathogenomics of novel virulence factors

The ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulence factors acting individually or together at different stages of infection. Discovering virulence factors of pathogenic bacteria is a key in understanding pathogenesis and for identification of targets for novel drugs and design of new vaccines. Recently, we have found that eukaryotic cystatins and stefins have been acquired and co-opted by a few bacteria (Figure 5). Bacterial cystatins and stefins could play an important role in self-defence or attack against host inflammatory and immune responses, by inhibiting cysteine cathepsins that are essential for host innate and acquired immunity. Our hypothesis is that some pathogenic bacteria have evolved independently by horizontal gene transfer a novel anti-immune strategy (similarly as eukaryotic parasites) to overcome host innate immunity. In order to demonstrate the biochemical activity of bacterial stefins, we expressed *Vibrio cholerae* stefin (VCA0935) and *Bacteroides fragilis* fusion inhibitor containing chagasin and cystatin domains (BF1388). We explored the inhibitory properties of recombinant proteins VCA0935 and BF1388 and determined their interaction constants with diverse cysteine proteases, cathepsins L, S, K, V, B and papain. Both VCA0935 and BF1388 were found to act as fast and tight binding inhibitors of endopeptidases cathepsins K, S, V, L and papain, however, their interaction with exopeptidase cathepsin B was several orders of magnitude weaker. Interestingly, the bacterial stefins inhibit the endopeptidase

activity of cathepsins S, K, L and V, which are all important players in the host adaptive and innate immunity. There are a very few cases where protease inhibitors have been shown to assist pathogens in invading the eukaryotic hosts by inhibiting host proteases. Stefins and cystatins with inhibitory spectra for papain family of cysteine proteases are especially suited to inhibit the numerous eukaryotic host cysteine proteases during infection. In this way, the bacterial stefins and cystatins could function in the invasion and dissemination of the pathogens.

Expert body of the Scientific Council at the Slovenian Research Agency selected our work, in which we traced the genesis and evolution of retroelement-derived multigene families of domesticated genes (J. Kokošar in D. Kordiš, *Mol. Biol. Evol.*, 30 (2013), 1015–1031), as the most important scientific achievement of the year 2013 in the field of Biochemistry and Molecular Biology.

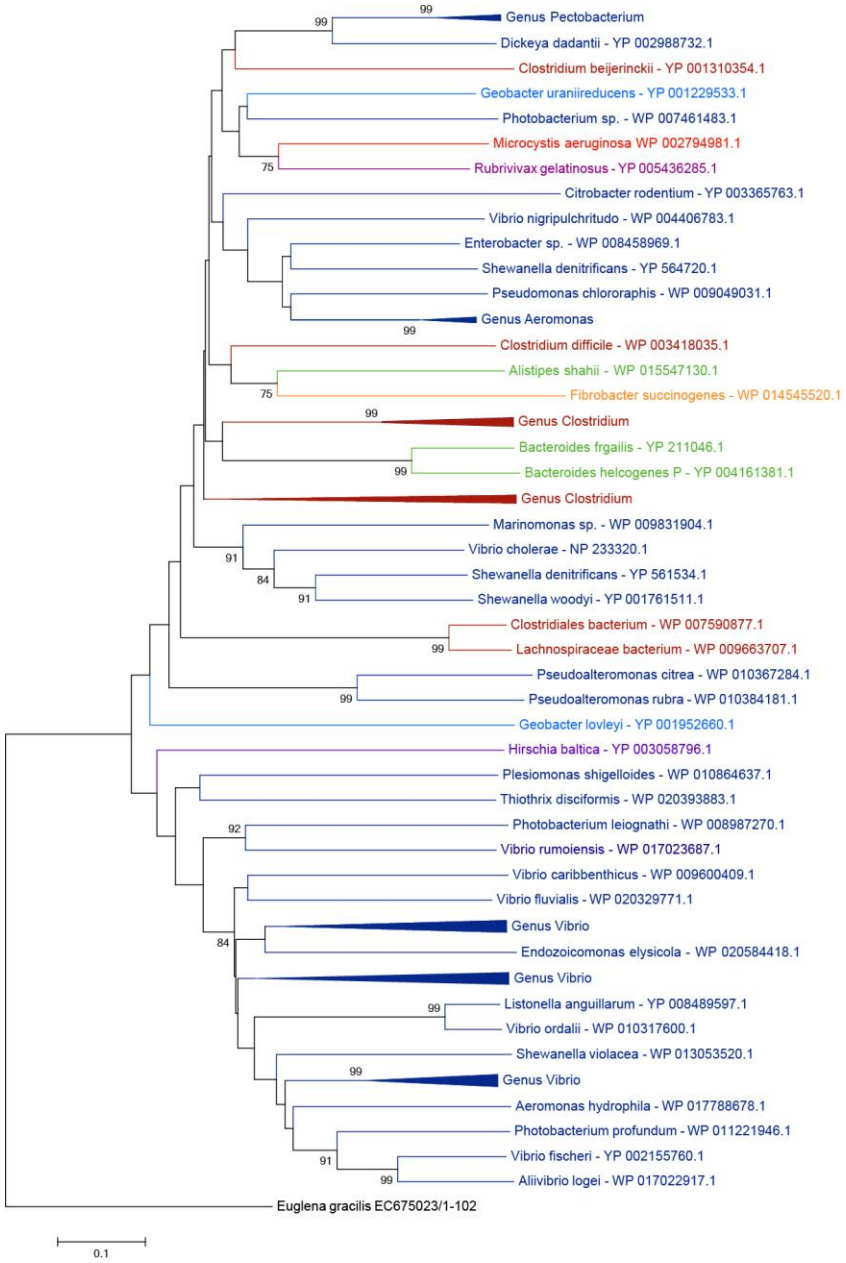

Figure 5: Bacterial representatives of the cystatin superfamily.

Other subjects

In 2014 we also worked on several projects out of the thematic scope of our department.

We collaborated intensively with the colleagues from the Department of Biology, the Biotechnical Faculty, University of Ljubljana, at elucidating the haemolytic activity of bovine erythrocytes with an ethanol extract of the *Aspergillus niger* mycelium (M. Novak et al., *Molecules*, 19 (2014), 9051–9069).

With colleagues from the Department of Biochemistry, Molecular and Structural Biology at the Jožef Stefan Institute we contributed to the understanding of the role of human stefin B in cellular response to protein aggregates with autophagy, by expressing it in *Saccharomyces cerevisiae* and identifying its genetic interactions (M. Polajnar et al., *PLoS One*, 9 (2014), e102500).

In collaboration with our colleagues rheumatologists from the University Medical Centre Ljubljana, we improved the isolation procedure for two human serum proteins that are essential for the diagnostics of the antiphospholipid syndrome (A. Artenjak et al., *J. Immunol. Res*., 2014 (2014), e195687).

With our colleagues from the Institute of Biochemistry, Medical Faculty, University of Ljubljana, we collaborated on the discovery of new substances with antifungal activity with tridimensional modelling of the structure and mapping of the active site of proteins from the family of fungal cytochrome P450 monooxygenases (P. Jawallapersand et al., *PLoS One*, 9 (2014), e107209).

Most important publications in 2014

1. Šribar, J. Oberčkal, J. and Križaj, I.: Understanding the molecular mechanism underlying the presynaptic toxicity of secreted phospholipases A_2 – an update. Toxicon, 89 (2014), 9–16
2. Brglez, V., Pucer, A., Pungerčar, J., Lambeau, G. and Petan, T.: Secreted phospholipases A_2 are differentially expressed and epigenetically silenced in human breast cancer cells. Biochem. Biophys. Res. Commun., 445 (2014), 230–235
3. Brglez, V., Lambeau, G. and Petan, T.: Secreted phospholipases A_2 in cancer: Diverse mechanisms of action. Biochimie, 107 (2014), 114–123
4. Leonardi, A., Sajevic, T., Kovačič, L., Pungerčar, J., Lang Balija, M., Halassy, B., Trampuš-Bakija, A. and Križaj, I.: Hemorrhagin VaH4, a covalent heterodimeric P-III metalloproteinase from *Vipera ammodytes ammodytes* with potential anti-tumour activity. Toxicon, 77 (2014), 141–155
5. Petschnigg, J., Groisman, B., Kotlyar, M., Taipale, M., Zheng, Y., Kurat, C.F., Sayad, A., Sierra, J.R., Mattiazzi Usaj, M., Snider, J., Nachman, A., Krykbaeva, I., Tsao, M.-S., Moffat, J., Pawson, T., Lindquist, S., Jurisica., I. and Stagljar, I.: The mammalian-membrane two-hybrid assay (MaMTH) for probing membrane-protein interactions in human cells. Nat. Methods, 11 (2014), 585–592