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 ($sPLA_2$s)

The major research topic of the department are $sPLA_2$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.

By the means of protein engineering and chemical synthesis we prepared several new molecular tools for characterising $sPLA_2$ binding proteins, searching for novel receptors, studying dynamics of translocation of $sPLA_2$s from external space into the cells, localization of $sPLA_2$s inside cells and their co-localization with already described binding proteins.

In 2012 we continued with the intensive research of the molecular mechanism of action of presynaptically neurotoxic $sPLA_2$s. As model $sPLA_2$s in our studies we used ammodytoxin (Atx) from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*), belonging to group IIA $sPLA_2$s, and also OS_2 from the venom of the Australian taipan (*Oxyuranus scutellatus scutellatus*), which is a group I $sPLA_2$. We have been trying to answer several key questions about the action of this group of neurotoxic enzymes. We were interested in the identification of the N-type $sPLA_2$ receptor in the presynaptic membrane of a motoneuron, which is crucial for the expression of neurotoxicity. To trace this receptor we decided to use OS_2 that binds to it with a 1000-fold higher affinity than Atx. In the scope of an international bilateral project Proteus with a research group from the Institute of Molecular and Cellular Pharmacology of the National Centre for Scientific Research (CNRS), Valbonne, France, we prepared large quantities of the recombinant wild-type OS_2 from the venom of the Australian taipan and its chimera with a similar, but non-toxic, OS_1 from the venom of the same snake. In the following, their photo-reactive derivatives will be prepared that are expected to enable the identification of the N-type receptor for $sPLA_2$s in mammals.

Following demonstrations that $sPLA_2$s can act also inside cells, their intracellular activity became a very attractive research topic of numerous research groups. In our group these investigations have been conveyed using different cellular models. In the past year we concluded an investigation of the molecular mechanism of action of Atx in yeast *Saccharomyces cerevisiae*. Based on the results of SGA (synthetic genetic array) analysis (Figure 1) and the analysis of the influence of cytosol-expressed Atx on the dynamics of sites of endocytosis in the plasma membrane (PM) of the yeast cell, we concluded that Atx significantly inhibits the process of endocytosis by inhibiting the function of amphiphysin. Amphiphysin is a protein which plays a key role in the final steps of the release of endocytotic vesicles from the PM. In the initial phase of Atx action on endocytosis, its binding to 14-3-3 protein, located on the PM at the sites where endocytic vesicles start to form, is important. Afterwards, Atx phospholipase activity is crucial for the blocking effect (M. Mattiazzi et al., *PLoS ONE*, 7 (2012), e40931). In the future we will test the hypothesis, based on the results obtained in yeast, about the way of inhibition of the process of endocytosis by $sPLA_2$s from the cytosol also in mammalian cells. Knowing how do neurotoxic $sPLA_2$s act may open new approaches to the regulation of endocytosis, which would be extremely important for human medicine. The functional-genomic approach to study the molecular mechanisms of action of animal toxins in the yeast cell, introduced by our group in the field of toxinology, obviously attracted considerable attention – the editor of Toxicon, the main

toxinology journal, invited us to prepare a review paper on this topic (M. Mattiazzi et al., *Toxicon*, 60 (2012) 558–571).

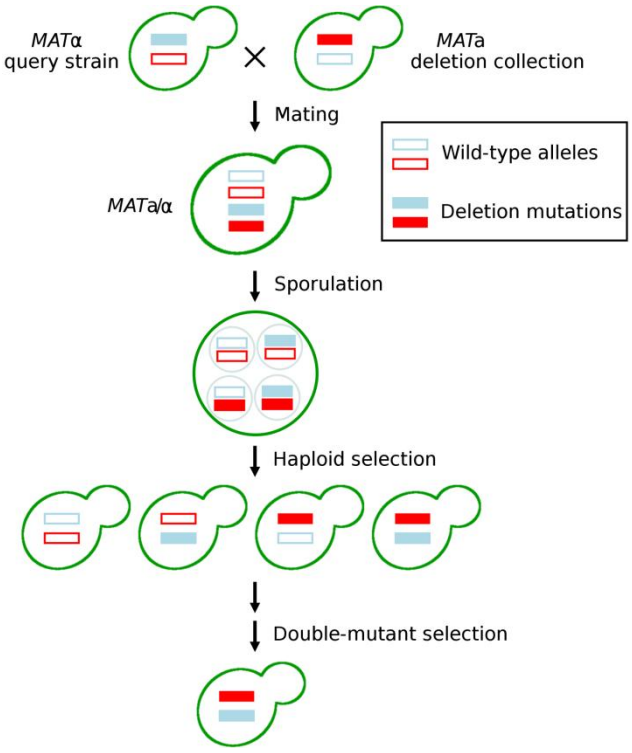

Figure 1: Schematic representation of the synthetic genetic array (SGA) analysis method. SGA is a technique that enables identification of the genetic interactions between genes in a systematic manner.

Intensive research of the intracellular action of Atx proceeded also by using two mammalian cell lines, mouse NSC34 and rat PC12 cells. Using confocal microscopy we observed the dynamics of internalization of Atx into the PC12 cells. Among other experiments in these cells, we also confirmed co-localization of Atx with mitochondria (Figure 2).

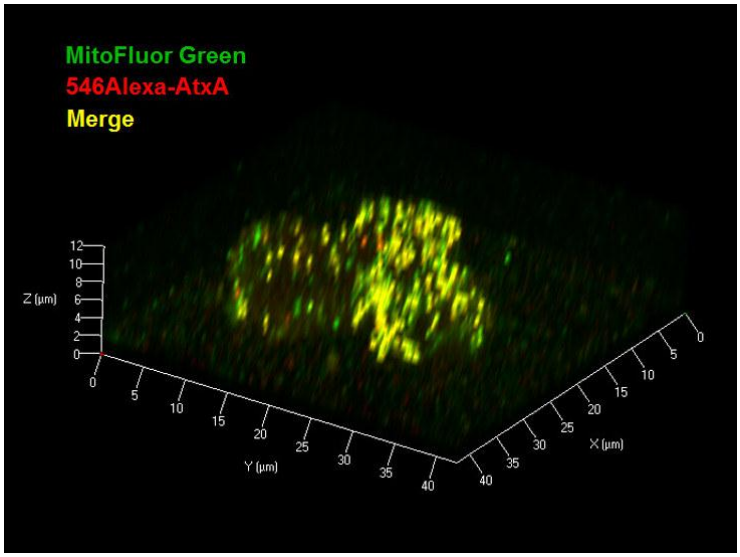

Figure 2: Co-localization of Atx and mitochondria in PC12 cells. Cells were incubated with fluorescently labelled AtxA (red signal) and a marker specific for mitochondria (green signal). Using confocal microscope at 100-fold magnification we acquired 13 optical slices. *In silico*, these were then assembled into a tridimensional image. Yellow signal corresponds to areas where AtxA and mitochondria are co-localized.

It is presumed that the interaction of certain $sPLA_2$s, including Atx, with calmodulin (CaM), a regulatory protein in the cytosol, is physiologically very important. Therefore, we study this

interaction with a particular interest. Aiming to observe dynamics of the interaction between Atx and CaM in the cell using a FRET method, we were developing fluorescence derivatives of both proteins in the past year. We are also very interested in a detailed structural analysis of the interaction between Atx (and homologous mammalian $sPLA_2$s) and CaM, as well as of the interaction of the $sPLA_2$–CaM complex with the phospholipid membrane, which will be accomplished by using protein NMR spectroscopy. To this end we prepared in the scope of a postdoctoral research project the recombinant ^{13}C- in ^{15}N-labelled Atx and CaM. In 2012, we already acquired the first NMR spectra. In the NMR studies we collaborate with two partner groups, the Bijvoet Centre from the Utrecht University, Netherlands, and the NMR centre from the National Institute of Chemistry, Ljubljana, Slovenia. With the already obtained results we succeeded to raise additional funds from the European project FP7-Bio-NMR to cover the expenses of the NMR measurements at the Utrecht University.

We concluded the bilateral project with Bulgarian colleagues from the Sofia University. In the scope of this project we looked for the differences in the mechanism of neurotoxic action of monomeric Atx from the venom of our subspecies of the nose-horned viper (*Vipera a. ammodytes*) and the two-chain vipoxin from the snake venom of the Bulgarian subspecies (*Vipera a. meridionalis*). The data obtained, leading to the conclusion that, in spite of the high structural identity between Atx and vipoxin, the mechanisms of their neurotoxic action substantially differ, are being prepared for publication.

There is a common problem in many of the early studies of isolated snake venom $sPLA_2$s – the results obtained may not be reliable, particularly when there is a reasonable doubt as to whether the toxins tested were completely pure. AtxA is the most toxic $sPLA_2$ of three isotoxins with presynaptic neurotoxicity of the nose-horned viper, with an LD_{50} of 21 mg/kg in mice. The toxicity of AtxA, purified from the viper's venom, has been confirmed by that of recombinant AtxA, prepared by protein engineering in a bacterial expression system of *Escherichia coli*. We have also re-evaluated the toxic potencies of two other isoforms, AtxB and AtxC, by using highly purified recombinant proteins. It has been shown that their intraperitoneal LD_{50}s determined as 960 mg/kg for AtxB and 310 mg/kg for AtxC significantly differ from the values previously reported for these isoforms isolated from the snake venom (P. Prijatelj-Žnidaršič and J. Pungerčar, *Toxicon*, 6 (2012), 642–643). Our results also point to an even more important role of the Tyr115/Ile116/Arg118/Asn119 cluster in neurotoxicity of Atxs and similar toxins than previously thought. At the end of the past year, we also completed and submitted for publication the study of a putative involvement of free arachidonic acid, released as a result of enzymatic activity of AtxA, in apoptotic changes of montoneuron-like cells.

Several $sPLA_2$ enzymes have been implicated in the pathology of cancer, with roles in either tumour promotion or inhibition, depending on the tissue and biochemical microenvironment of the tumour involved. The group X $sPLA_2$ ($sPLA_2$-X) efficiently released fatty acids and lysophospholipids from various cells and stimulates colon cancer cell proliferation in an enzymatic activity-dependent manner. In order to elucidate a possible role of $sPLA_2$-X in breast cancer, we analysed the effects of exogenously-added recombinant $sPLA_2$-X on the viability, proliferation and survival of model breast cancer cell lines with different tumourigenicity. Already low nanomolar concentrations of exogenously added recombinant $sPLA_2$-X stimulated the proliferation of highly invasive breast cancer cells, but decreased the viability of weakly and moderately tumorigenic cells. The positive effect on breast cancer cell proliferation was confirmed with ectopically expressed $sPLA_2$-X as well. Importantly, the proliferative effect was strictly dependent on PLA_2 enzymatic activity, as it was completely abolished by the pan-$sPLA_2$ inhibitor varespladib. Furthermore, the critical role of enzymatic activity was confirmed in experiments with exogenously-added enzymatically inactive active-site mutant of $sPLA_2$-X as well as with forcible expression of the same mutant in breast cancer cells. Since the positive effect on cell proliferation was more significant in starved cells, we asked if $sPLA_2$-X exerts also an anti-apoptotic role in severely starved cells. Indeed, exogenously added as well as ectopically

expressed $sPLA_2$-X prevented serum-withdrawal induced cell death of the highly invasive breast cancer cells. The effect was strictly dependent on $sPLA_2$ enzymatic activity and was most significant in the highly invasive MDA-MB-231 and T-47D breast cancer cells and absent in the weakly tumourigenic MCF-7 cells (Figure 3).

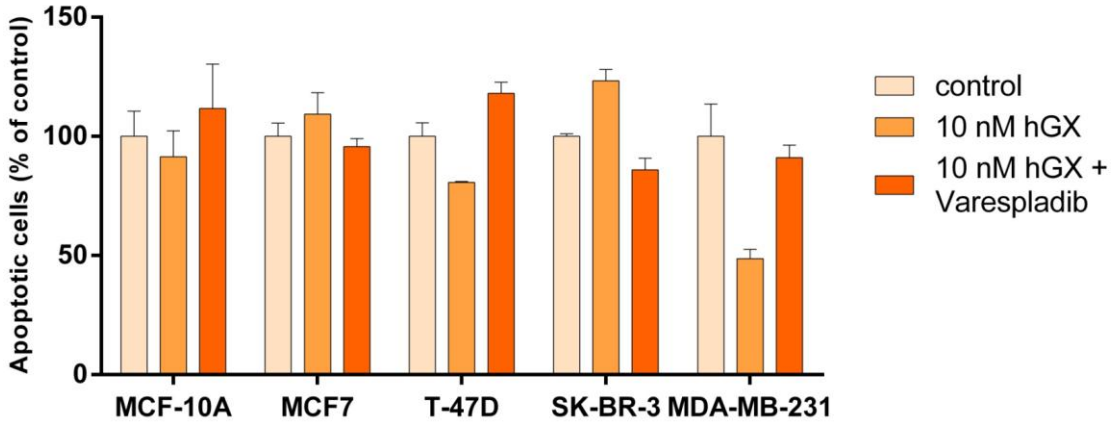

Figure 3: Human $sPLA_2$-X conveys a significant survival advantage only to the highly invasive breast cancer cell line MDA-MB-231. Cells of five breast cancer cell lines were serum-starved and treated in serum-free medium with $sPLA_2$-X for 96 h before flow cytometric analysis of apoptosis. The inhibition of enzymatic activity $sPLA_2$-X with varespladib completely abolished its effect on cell survival.

It has been shown previously that exogenously added oleic acid prevents serum-withdrawal induced apoptosis most significantly in MDA-MB-231 and T-47D cells. The pro-survival action of oleic acid was linked to the particular ability of these two cell lines to accumulate high amounts of triglycerides in lipid droplets. Since oleic acid is one of the major products of $sPLA_2$-X cell membrane hydrolysis, we reasoned that $sPLA_2$-X might affect lipid cycling and accumulation as well as prolong the survival of breast cancer cells. Indeed, we found that $sPLA_2$-X induced lipid droplet formation in the serum-starved as well as in proliferating cells in an enzymatic activity-dependent manner. Using a range of cell signalling and lipid metabolism inhibitors we found that fatty acid activation, mitochondrial fatty acid oxidation and AMP-activated protein kinase, a key regulator of cellular lipid metabolism, are involved in both the pro-survival and lipid droplet-inducing effects of $sPLA_2$-X. The pro-survival and anti-apoptotic signalling is associated with changes in lipid storage and fatty acid metabolism. The effects of $sPLA_2$-X on the growth and survival of breast cancer cells reveal previously unknown connections between $sPLA_2$-mediated fatty acid release and alterations of lipid metabolism in cancer.

Another aspect of our research on the involvement of human $sPLA_2$s in disease is the mechanisms of regulation of $sPLA_2$ gene expression. Since tumours depend on aberrant epigenetic modifications that enable their growth and survival, we wanted to determine the involvement of DNA methylation and histone acetylation in the regulation of $sPLA_2$ expression in a panel of cell line models of breast cancer. Treatment of cells with a DNA-methyltransferase inhibitor led to a significant increase in the expression of group IIA, III and X $sPLA_2$s, indicating that DNA hypermethylation is responsible for $sPLA_2$ silencing in breast cancer cells. Bisulphite sequencing of $sPLA_2$ promoter regions and treatment of cells with transcription factor inhibitors suggested that Sp1, estrogen receptor alpha (ER-α), retinoic acid receptor alpha (RAR-α) and sterol segulatory element-binding protein (SREBP) transcription factors are crucial for $sPLA_2$ silencing by hypermethylation. Furthermore, the expression of group IIA, III and X $sPLA_2$s was restored in cells treated with a histone deacetylase inhibitor, particularly in the most tumourigenic

cell line used, and it was even further augmented upon inhibiting both cellular DNA methyltransferases and histone deacetylases. Our results clearly show that both DNA hypermethylation and histone acetylation are involved in $sPLA_2$ gene silencing in breast cancer cells, particularly in highly tumourigenic and invasive cells, and suggest a functional importance of these enzymes in malignant cell transformation.

Other pharmacologically active components from natural toxins

In the past year we continued the intensive study of the components of the nose-horned viper venom that affect the coagulation of blood – haemostasis. In the scope of a national research project we systematically evaluated with our partners from the University Medical Centre Ljubljana, Division of Pediatrics, the influence of isolated venom components on different parts of the human haemostatic system. In this process we selected several venom proteins for further in depth analysis. We concluded with the experimental work on description of one of the major haemorrhagic molecules in the venom, homodimeric metalloproteinase (SVMP) VaH3 (Figure 4), and practically finished the analysis of another haemorrhagic SVMP, VaH4, which is a heterodimeric protein. The results are now being prepared for publication. Due to conspicuous achievements in research of haemostasis and haemostasis-related pathologies we had been invited to give an interview that was published in a prominent American journal *Circulation* (I. Križaj, *Circulation*, 126 (2012), f5–f6).

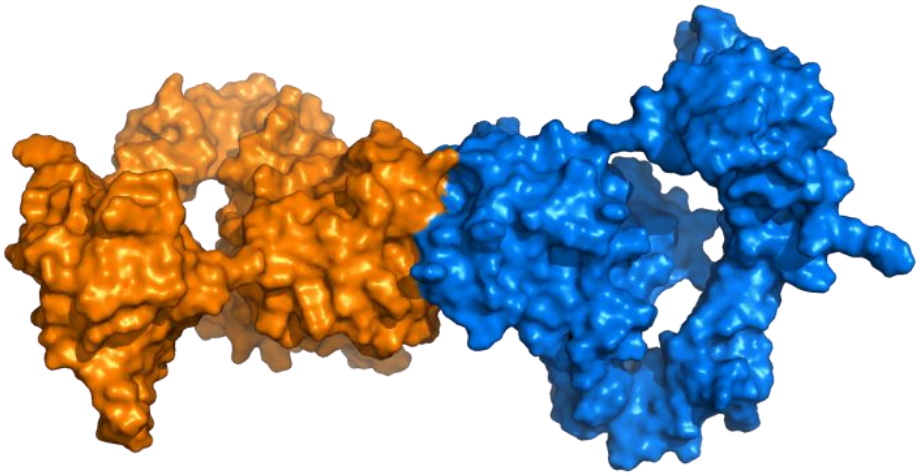

Figure 4: Tridimensional model of VaH3, the haemorrhagic snake venom metalloproteinase from the venom of the nose-horned viper. The model represents a valuable instrument to study structure-function relations of ADAM/ADAMTS, a homologous family of mammalian proteins with high therapeutic potential.

For many years we have been collaborating successfully with our colleagues from the Institute of Immunology in Zagreb, Croatia, on the development of procedures for production of more effective antivenoms and methods for the testing of their quality. In 2012 we published together a study which reported that using the standard antivenom quality test on mice one cannot establish the content of the antibodies in the antiserum that are able to neutralize haemorrhagins in the nose-horned viper's venom. In other words, the standard mouse test is inadequate for human medicine (T. Kurtović et al., *Toxicon* 59 (2012), 709–717).

As one of the 20 partners on the EU 6FP integrated project "Conco" we have been involved in the analysis of the genome, transcriptome and venom proteome of the piscivorous marine snail

Conus consors and related snails. In 2012, when this project was concluded, we succeeded to publish two papers of the proteomic analysis of the high molecular mass (HMM) protein components isolated from the so called “dissected” and “injected” venoms of *C. consors* (Figure 5). In this analysis we discovered proteins that define new protein families of unknown biological function. It is particularly interesting that some of these new conoproteins are highly represented and appear to be present exclusively in cone snails (genus *Conus*). Derived complete or at least partial sequences of these structurally unique proteins will enable study of their biological roles. The analysis of venom duct and salivary gland EST libraries has also demonstrated differential expression sites of *C. consors* venom HMM proteins. Collectively, our results enabled better understanding of the biological role of the HMM in the venom of cone snails (A. Leonardi et al., *Journal of Proteome Research*, 11 (2012), 5046–5058; A. Violette et al., *Marine Drugs* 10 (2012), 258–280).

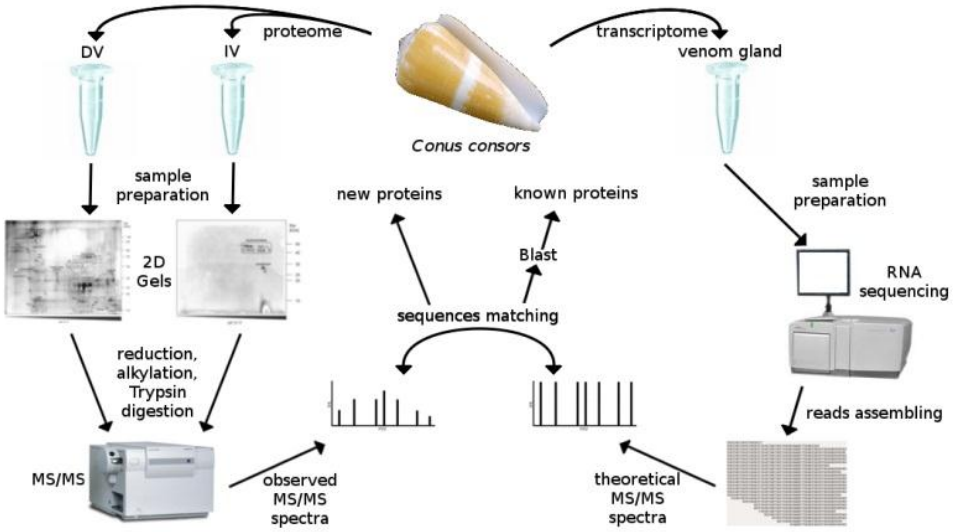

Figure 5: Workflow of *Conus consors* snail venom proteomics analysis. The study opened a way to understanding of biological roles of the high molecular mass proteins in the venom. We discovered also new families of proteins and the deduced amino acid sequences will enable clarification of their functions. The figure is reproduced from A. Leonardi et al., *Journal of Proteome Research*, 11 (2012), 5046–5058.

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

Obesity and the resulting type 2 diabetes are a pressing health-related problem of today’s societies, both in developed and developing countries. Biology of the changes in metabolism leading to obesity and diabetes is, however, not well understood. Insulin is the most important hormone to regulate sugar metabolism, but other hormones, such as adiponectin, play additional roles and also affect the metabolism-related disorders like diabetes. Specifically, adiponectin suppresses type 2 diabetes.

Zinc is an essential mineral that has also been implicated in the development of diabetes. It is required for the formation of insulin hexamer, the storage form of the hormone, and is thus important for synthesis, storage, proper conformation and excretion of insulin from the pancreatic β-cells. Zinc depletion in humans can therefore lead to insulin production and

secretion disorders, and hyperglycemia results in increased secretion and decreases in total body zinc. The connection between diabetes and zinc is complex and still without a clear cause and effect relationship. Using yeast as a model system we have analyzed the genetic interactions of zinc depletion and overload, and in this context we additionally analyzed the role of the homologues of adiponectin receptor, yeast Izh proteins. It has been shown that the effects of Izh deficiency and zinc depletion overlap, and that both exert a response typical of membrane fluidity changes. Using novel bioinformatics tools, we identified a modular nature of cellular responses to environmental or genetic perturbations. We thus demonstrated that Zn^{2+} concentration modulation is potentially useful in preventing and possibly also treating diabetes. However, rather than having a direct, diabetes-related target, it elicits an effect which resembles the inverse of the effects that occur as a consequence of non-healthy lifestyle leading to diabetes.

Red-spotted newt (*Notophthalmus viridescens*) specimens from various locations in Canada and USA were analyzed for the presence of tetrodotoxin (TTX) and its analogues (M. Yotsu-Yamashita et al., *Toxicon*, 59 (2011), 257–264). Considerable individual variations in toxin levels were found within and among populations. TTX and its analogues were absent in efts and adults from the northernmost locations of the newt, and in adults from Florida. Newts kept in captivity for several years and reared on toxin-free diet lost their toxicity. Bayesian and maximum likelihood phylogenetic analysis of specimens from the various populations using three phylogenetic markers (COI, ND2 and 16S RNA) revealed that populations from the northern states of the USA and Canada are genetically homogenous, whereas the newts from Florida exhibited a much higher level of genetic divergence. This analysis has demonstrated that TTX-bearing populations are not genetically separated from those that lack TTX. Therefore, an exogenous source of TTX in the newts either *via* the food chain or its synthesis by symbiotic bacteria was suggested to explain the high variability and lack of TTX in certain populations.

In the invited review we summarized the current understanding of intron gain in mammals (D. Kordiš and J. Kokošar, *International Journal of Evolutionary Biol*ogy, 2012 (2012), e278981). Domesticated genes, originating from retroelements or from DNA-transposons, constitute an ideal system for testing the hypothesis on the absence of intron gain in mammals. Since single-copy domesticated genes originated from the intronless multicopy transposable elements, the ancestral intron state for domesticated genes is zero. A phylogenomic approach has been used to analyse all domesticated genes in mammals and chordates that originated from the coding parts of transposable elements. A significant amount of intron gain was found only in domesticated genes of placental mammals, where more than 70 cases were identified (Figure 6). *De novo* gained introns show clear positional bias, since they are distributed mainly in 5' UTR and coding regions, while 3' UTR introns are very rare. In the coding regions of some domesticated genes up to 8 *de novo* gained introns have been found. Surprisingly, the majority of intron gains has occurred in the ancestor of placental mammals. Domesticated genes thus represent an excellent system to study the mechanisms that allow the entry of newly formed introns in genes of placental mammals.

In an invited review we summarized the current understanding of the repetitive landscape of sauropsid genomes (D. Kordiš, *Evolutionary Biology: Mechanisms and Trends*, (2012); Heidelberg, New York, Dordrecht, London. Springer, pp. 243–263). Investigations of transposable elements (TEs) in sauropsid genomes over the last four decades have provided an insight into the TE repertoires of all major extant sauropsid lineages. Invaluable information concerning the diversity, activity, and repetitive landscapes in sauropsids has emerged from the analyses of the chicken and *Anolis* (lizzard) genomes and other preliminary reptilian genome sequencing projects. Avian and reptilian genomes differ significantly in the classes of TEs present, their fractional representation in the genome and by the level of TE activity. While lepidosaurian genomes contain many active TE families, the extant avian genomes have few active TE lineages. Most reptilian genomes possess quite rich TE repertoires that differ

considerably from those of birds and mammals. In sauropsid genomes, TEs have been active for more than 300 millions of years, and as such have had a large impact on the genetic diversity and genome architectures.

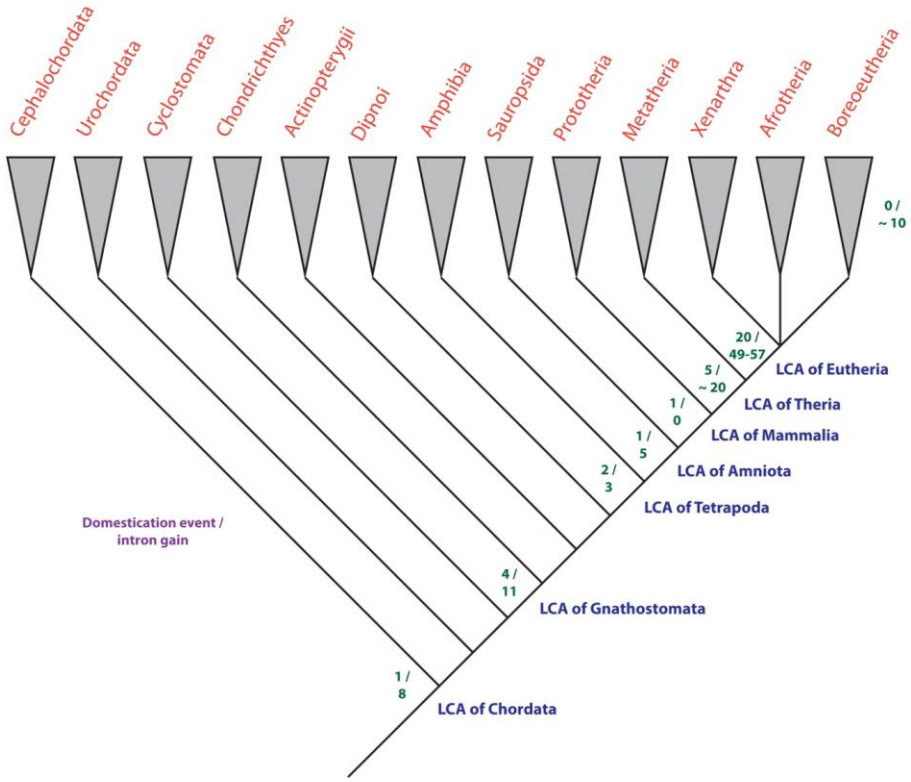

Figure 6: Numbers of transposable element-derived gene domestication events and intron gains mapped on the chordate phylogenetic tree. In the superorder *Boreoeutheria* some additional intron gains have occurred. The figure is reproduced from D. Kordiš and J. Kokošar (2012), *International Journal of Evolutionary Biol*ogy, 2012 (2012), 278981-1-278981-7.

Other subjects

In the year 2012 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, Biotechnical faculty, University of Ljubljana. With a structural analysis we participated at the determination of the mode of action of membrane-active proteins from the mushroom *Pleurotus ostreatus*. The conclusion of this study was that pleurotolysin B obligatory require a presence of another protein ostreolysin A to form a pore in the membranes rich in cholesterol and sphingomyelin. The publications are in preparation. Together with the same group we also prepared a review paper about the use of pore-forming toxins at sensing and labelling of membrane microdomains (M. Skočaj et al., *Current Medicinal Chemistry*, in press). Very important joint project in 2012 was dedicated to developing of an original approach against bacterial infections. In evolvement of the resistance of bacteria against antibiotics their SOS system is of a crucial importance. The key role in the bacterial SOS response is played by a complex formed between a single-stranded DNA (ssDNA) and two bacterial proteins, RecA and LexA. Based on experimental data we build

a tridimensional model of the complex ssDNA–RecA–LexA which will enable a design of substances to prevent development of bacterial resistance to antibiotics. The publication is in preparation.

In collaboration with the NMR Centre from the Utrecht University we prepared a chapter in a scientific monograph (L. Kovačič in R. Boelens, *NMR of Biomolecules: Towards Mechanistic Systems Biology*, (2012); Weinheim, Chichester. Wiley-VCH, pp. 239–252). In addition, by performing surface plasmon resonance (SPR) measurements, we studied together with this group the mechanism of binding of structure-specific endonuclease ERCC1/XPF on DNA in the process of its repair.

Most important publications in 2012

1. Mattiazzi, M., Sun, Y., Wolinski, H., Bavdek, A., Petan, T., Anderluh, G., Kohlwein, S.D., Drubin, D., Križaj, I. and Petrovič, U. (2012): A neurotoxic phospholipase A_2 impairs yeast amphiphysin activity and reduces endocytosis. *PLoS ONE* 7, e40931.
2. Jenko-Pražnikar, Z., Petan, T. and Pungerčar, J. (2012): Ammodytoxins efficiently release arachidonic acid and induce apoptosis in a motoneuronal cell line in an enzymatic activity-dependent manner. *NeuroToxicology*, in press.
3. Leonardi, A., Biass, D., Kordiš, D., Stöcklin, R., Favreau, P. and Križaj, I. (2012): *Conus consors* snail venom proteomics unveils functions, pathways and novel families involved in its venomic system. *J. Proteome Res.* 11, 5046–5058.
4. Violette, A., Leonardi, A., Piquemal, D., Terrat, Y., Biass, D., Dutertre, S., Noguier, F., Ducancel, F., Stöcklin, R., Križaj, I. and Favreau, P. (2012): Recruitment of glycosyl hydrolase proteins in a cone snail venomous arsenal: further insights into biomolecular features of *Conus* venoms. *Mar. Drugs* 10, 258–280.
5. Mattiazzi, M., Petrovič, U. and Križaj, I. (2012): Yeast as a model eukaryote in toxinology: a functional genomics approach to the studies of the molecular basis of action of pharmacologically active molecules. *Toxicon* 60, 558–571.