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 molecular mechanisms of action of 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.

In the past year the most intensive research has been performed in the area of molecular mechanism of action of presynaptically neurotoxic $sPLA_2$s. As the model $sPLA_2$ in these studies we used ammodytoxin (Atx) from the venom of the nose-horned viper (*Vipera ammodytes ammodytes*). With particular intensity we have been trying to find answers about the identity of the specific Atx receptor in the presynaptic membrane of the motoneuron, the so called N-type $sPLA_2$ receptor, and about intracellular events following translocation of the toxin through the neuronal plasma membrane. In the N-type $sPLA_2$ receptor studies we joined forces on a bilateral project Proteus with the renowned group from the Institute for Molecular and Cellular pharmacology CNRS (Institut de Pharmacologie Moléculaire et Cellulaire CNRS) from Valbonne, France. By the means of protein engineering and chemical synthesis we almost finished preparation of molecular tools to dynamically follow localization of Atx in cells and its interactions with binding proteins. Calmodulin (CaM) is one of the binding proteins of Atx in the cytosol of the nerve cell. Complexed to CaM, Atx becomes completely stable in a reducing environment, such as the cytosol, and its enzymatic activity substantially increased. Why such pathophysiologically potentially very important effects occur we started to study within the scope of a new postdoctoral project. Using the recombinant DNA technology, we prepared in 2011 isotope-labelled Atx and CaM, which we need to study the interactions between these two proteins and interactions of the complex Atx–CaM with the phospholipid membrane by the nuclear magnetic resonance (NMR). Our partner groups in the NMR studies are prominent NMR centres, Bijvoet Centre from Utrecht University, Netherlands, and the centre from the National Institute of Chemistry, Ljubljana, Slovenia. In the bilateral project with Bulgarian colleagues from the Sofia University, we discovered differences in the mechanism of neurotoxic action of monomeric Atx from the venom of our subspecies of the nose-horned viper (*V. a. ammodytes*) and the two-chain vipoxin from the venom of Bulgarian subspecies of the snake (*V. a. meridionalis*). Interestingly, in spite of high structural identity between Atx and vipoxin, the mechanisms of their neurotoxic action substantially differ.

Very important steps in searching the answer about the mode of action of this group of lethal neurotoxins have been made by using Atx. Invited by the Editor of Toxicon, a leading journal in the field of toxinology, the review article describing comprehensively experiments performed on Atx and with Atx was prepared (I. Križaj, *Toxicon*, 58 (2011) 219–229). This article was the introductory one into a new series of review articles, Classic Toxins Review, in Toxicon, which we considered as a special recognition to achievements of our group. In a review article we presented the results of our past and up-to-date research about the relationships between the structure and function of neuro- and myotoxic $sPLA_2$s from the venom of the nose-horned viper (J. Pungerčar et al., *Acta Chimica Slovenica*, 58 (2011), 660–670).

Our studies on the involvement of endogenous mammalian $sPLA_2$s in breast cancer have continued in the year 2011. Ten structurally distinct $sPLA_2$ enzymes are known in humans

displaying diverse roles in a variety of physiological and pathophysiological processes owing to their enzymatic action on cellular and non-cellular phospholipids, downstream effects of the products of their hydrolysis or due to interactions with specific binding proteins. In mammals, $sPLA_2$s are involved in lipid digestion and homeostasis, inflammatory and immune response, acute and chronic airway disorders, atherosclerosis, host defence against infections, and recently they have also been implicated in cell proliferation, apoptosis and cancer. Due to their very low phospholipid-head group and acyl-chain specificity, their diverse tissue and cell expression patterns, and their ability to act intra- and extracellularly, they often display apparently contradictory biological roles, *e.g.* cell growth promotion or inhibition. Indeed, we have previously shown that a particular $sPLA_2$ may induce cell proliferation, apoptosis and neurotoxicity in different cellular environments. Recent studies have associated the expression of several $sPLA_2$s with the pathology of colorectal and prostate cancers, with roles in either tumour promotion or inhibition, depending on the tissue and biochemical microenvironment of the tumour involved. Their involvement in cancer biology might be related to their role in liberating arachidonic acid (AA) and lysophospholipids, such as lysophosphatidylcholine (LPC), from membrane phospholipids, which influence cell proliferation, survival and angiogenesis. AA is an important substrate for intracellular biochemical pathways that generate eicosanoids, potent autocrine and paracrine lipid mediators including the mitogenic prostaglandins (PG), which have been associated with a number of malignancies, including breast cancer. On the other hand, LPC can be converted to lysophosphatidic acid (LPA), which is a potent lipid mediator known to induce cell proliferation, survival and migration, critical requirements for cancer progression. Additionally, elevated expression of several important enzymes involved in eicosanoid metabolism, including cyclooxygenase-2 (COX-2) that catalyses the first step of AA conversion to various PGs, is a hallmark of different malignancies, including colorectal, prostate and breast cancer. However, the clinical use of non-steroidal anti-inflammatory drugs (NSAIDs) including specific COX-2 inhibitors in cancer prevention and treatment, as well as in pain and inflammation relief, is associated with some serious adverse effects. Therefore, considering their importance in regulating the availability of AA and lysophospholipids for downstream biosynthetic pathways that produce a variety of important lipid mediators, which influence crucial determinants of cancer initiation and progression, $sPLA_2$s are promising new targets for cancer prevention and therapy.

In our recent initial study of the involvement of $sPLA_2$s in breast cancer, we have determined the expression profile of all $sPLA_2$ family members in human breast cancer cell models representing different stages in the progression of the disease. Using a validated method for quantitative PCR expression analysis of the whole set of human $sPLA_2$s, we identified several differentially expressed $sPLA_2$s in breast cancer cells, namely the group IIA, III, V and X enzymes. For example, the group IIA $sPLA_2$ was overexpressed in moderately to highly invasive cell lines, while the group X enzyme ($sPLA_2$-X) was overexpressed in weakly and moderately invasive cells, but its expression was not detected in highly invasive and tumourigenic cell lines. In our latest studies in 2011, we have found that the expression of some $sPLA_2$s is upregulated by inflammatory cytokines, such as IL1-ß and TNF-α, in breast cancer cells. Furthermore, treatment of cells with a DNA demethylating agent resulted in significant increase in the expression of several $sPLA_2$ isoforms, indicating that the regulation of $sPLA_2$s in breast cancer involves epigenetic silencing by DNA hypermethylation. The differential expression patterns of $sPLA_2$s, regulation by inflammatory cytokines and epigenetic silencing suggest a potential role for these enzymes in human breast cancer and indicate that different $sPLA_2$s may have distinct roles at different levels of progression of the disease. Indeed, our gain-of-function studies, performed by ectopically over expressing $sPLA_2$-X in the highly invasive cell line MDA-MB-231 (Figure 1), show that the enzyme promotes breast cancer cell survival by increasing their proliferation rate and viability. A similar effect was observed after exogenous addition of low nanomolar concentrations of recombinant $sPLA_2$-X to the highly invasive breast cancer cells.

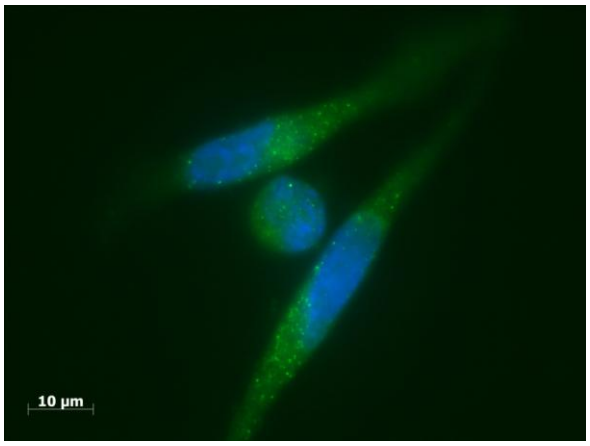

Figure 1. Immunofluorescently labelled $sPLA_2$-X, transiently expressed in breast cancer cells MDA-MB-231. Cell nuclei stained by a blue fluorescence dye. Green punctuate fluorescence stems from $sPLA_2$-X in the cytoplasm. The photograph was taken using epifluorescence microscope at 100-fold magnification.

Interestingly, $sPLA_2$-X also reduced the level of spontaneous apoptosis *in vitro* (Figure 2), suggesting a novel role for $sPLA_2$-X in promoting breast cancer cell survival. Therefore, our results focused on the regulation of $sPLA_2$ expression and their influence on breast cancer cell growth are very promising and suggest a novel protumorigenic role for the group X $sPLA_2$, which might be a consequence of different mechanisms of action affecting apoptosis and cell proliferation.

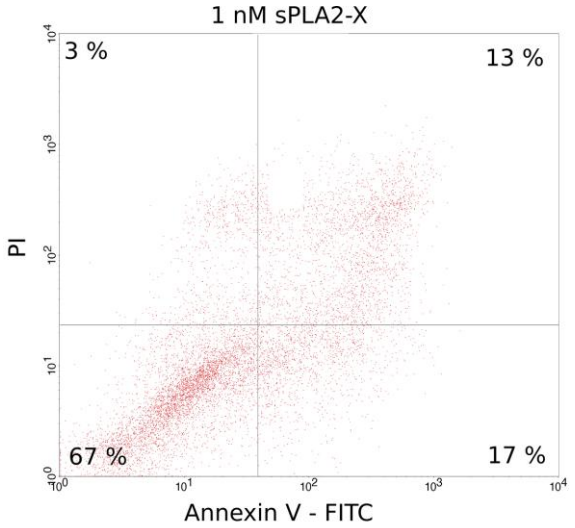

Figure 2. Cell death analysis by flow cytometry following the exposure of the breast cancer MDA-MB-231 cells to nanomolar concentration of recombinant $sPLA_2$-X. Viable cells (bottom left), cells in the early apoptotic phase (bottom right), cells in the late apoptotic phase (top right) and necrotic cells (top left).

In the past year, in the research of the role of endogenous $sPLA_2$s in the peripheral nervous system, we used cellular models of motor neurons to study (intra)cellular localization of exogenously added human $sPLA_2$-X and snake presynaptically neurotoxic $sPLA_2$, ammodytoxin A (AtxA). In the context of these studies, we first prepared the recombinant cysteine mutants with a single-site substitution N79C in the molecules of both $sPLA_2$s that were subsequently directly tagged with a fluorescent marker. The latter were exogenously added to the cell media and we monitored their internalization into a cell line of mouse motor neurons and into a heterologous tissue co-culture, prepared by the explants of rat embryonic spinal cord and human skeletal (striated) muscle cells. We found that AtxA entered the mouse motoneuronal cells by using the internalization pathway of human $sPLA_2$-X, although their intracellular colocalization with mitochondria and lysosomes showed certain important differences between the two $sPLA_2$s. The results were compared with those obtained in the tissue co-culture of rat

spinal cord explants and human muscle cells, and again we encountered certain differences in the localization of both $sPLA_2$s. Fluorescence microscopy analysis has shown that after the addition human $sPLA_2$-X is located primarily in neurites, whereas AtxA is specifically localized to the neuromuscular junctions. To analyse the effect of $sPLA_2$-X on the tissue co-culture, we treated the latter with different concentrations of $sPLA_2$-X and monitored their effect on the formation of functional neuromuscular junctions in the heterologous co-culture. We found that the addition of $sPLA_2$-X, depending on its concentration, has a significant influence on, i.e. modulates, the formation of neuromuscular junctions. A part of the results was obtained in collaboration with Dr. Tomaž Marš from the Institute for Pathophysiology, Medical Faculty, University of Ljubljana, and also presented at the international scientific conference SiNAPSA Neuroscience 2011 in Ljubljana.

In a review paper we presented a comprehensive overview of $sPLA_2$ binding proteins (J. Šribar and I. Križaj, *Acta Chimica Slovenica*, 58 (2011), 678–688). Novel interactors of these proteins are progressively discovered and it has become evident that many pathophysiological actions of $sPLA_2$s are linked not only to their enzymatic activity but also to their ligand function (Figure 3).

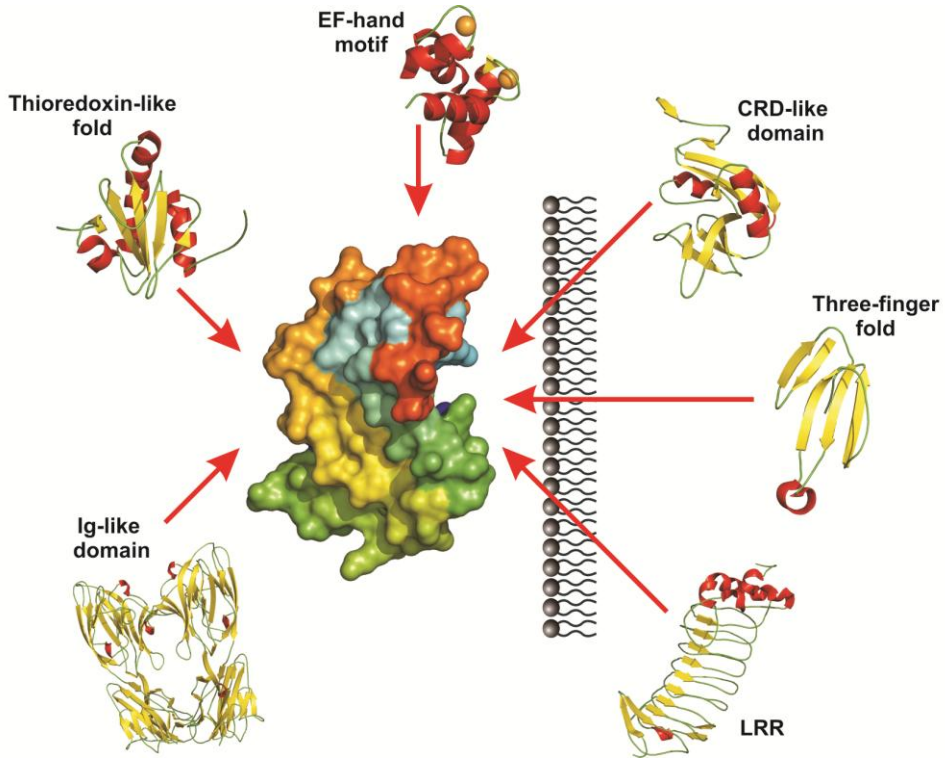

Figure 3. Secreted PLA_2s interact with a plethora of structurally different proteins. Some of these, containing well-defined structural motifs, are shown. CRD: carbohydrate recognition domain; Ig: immunoglobulin; LRR: leucine-rich repeat. The figure is reproduced from J. Šribar in I. Križaj, *Acta Chimica Slovenica*, 58 (2011), 678–688.

In the scope of a bilateral project with the researchers from the Institute of Immunology in Zagreb, Croatia, we continued our efforts to prepare efficient antiserum towards the nose-horned viper venom. We find out that methods for quantification of Atx content in the venom clearly differentiate between high and low immunogenic venoms of the nose-horned viper (B. Halassy et al., *Comparative Biochemistry and Physiology, Part C*, 153 (2011), 223–230).

Other pharmacologically active components from natural toxins

In 2011 we continued the intensive study of the components of the nose-horned viper venom that affect the coagulation of blood – haemostasis. Within this topic we also published a comprehensive review article (T. Sajevic et al., *Toxicon*, 57 (2011), 627–645) which was the seventh most downloaded paper of the journal at the end of the last year. We were able to obtain financing and started a new basic research project in 2011 in this area of our research. With our partners from the University Medical Centre Ljubljana, Division of Pediatrics, we evaluated the influence of venom fractions on different components of human haemostatic system. In collaboration with our colleagues from the Institute of Immunology in Zagreb, Croatia, we concluded part of our project by publishing a paper reporting on the characterisation of a potently hemorrhagic metalloprotease (MP) from the nose-horned viper venom, ammodytagin (T. Kurtović, et al., *Toxicon*, 58 (2011), 570–582). Ammodytagin represents the first dimeric MP described in this venom. A very important result brought by this paper was that it is possible to completely neutralize hemorrhagic activity of the whole venom by the antiserum raised only against ammodytagin. Preparation of a new cDNA library from the nose-horned viper venom glands has been initiated in 2011 which main goal was to isolate a full-length mRNA transcipt encoding ammodytagin.

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. At the beginning of 2011 we organized a very successful meeting of the project consortium at Brdo near Kranj.

Venoms of the marine cone snails consist of numerous proteins and peptides showing a wide variety of biological activities, such as their effects on ion channels and receptors. Conopeptides acting on neuronal nicotinic acetylcholine receptors (nAChRs) belong to several peptide superfamilies including the recently described αD-conopeptides which are homodimers of identical peptides with 47–49 amino acids. It has been demonstrated that αD-conopeptides specifically block mammalian neuronal nAChRs of the α7, α3β2 and α4β2 subtypes in nanomolar concentrations. Among the venom glands of 27 *Conus* species analyzed by cDNA cloning, precursors of αD-conopeptides were identified in four species only: *C. betulinus, C. capitaneus, C. mustelinus* and *C. vexillum*. Phylogenetic analysis of the relationships among the αD-conopeptides revealed that they belong to clades that are characterized by an AVV- and EMM-motif in the signal peptide sequence (Figure 4). The distribution of the αD-conopeptides in the *Conus* species is very limited. The overall dominance of these peptides and the low abundance or even lack of small α-conopeptides in the venoms of these *Conus* species may suggest that αD-conopeptides are an adaptation to a specific type of prey, *i.e.* marine worms (D. Mebs et al., *Acta Chimica Slovenica*, 58 (2011), 730–734).

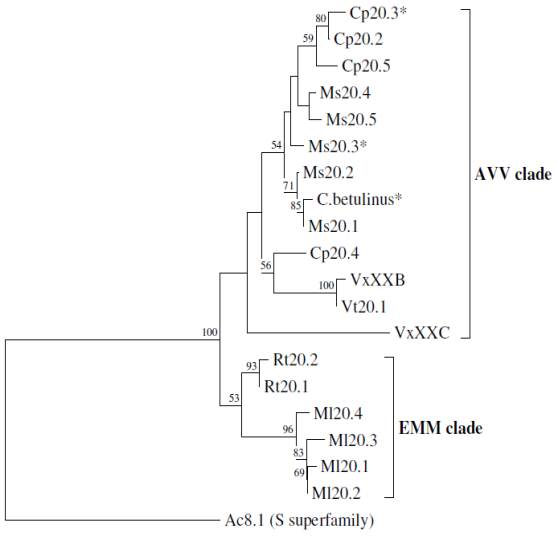

Figure 4. Phylogenetic tree of the αD-conopeptides. The figure is reproduced from D. Mebs et al., *Acta Chimica Slovenica*, 58 (2011), 730–734.

We performed phylogenetic and biogeographic analyses of diverse Indo-Pacific and endemic South African venomous cone snails (S. Kauferstein et al., *Toxicon*, 57 (2011), 28–34). Phylogenetic analysis of the 16S RNA from numerous *Conus* species has clarified the evolutionary position of the endemic South African *Conus* species and provided the first evidence for their close genetic relationship.

Experimentally, comparative proteomic analysis of the high molecular mass protein components in the cone snail venom duct and in its injected venom has been concluded. Part of our results is just close to the final acceptance for publication in *Marine Drugs*, whereas the main part of the results is still in preparation for publication.

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. In 2011, we have brought to an end a landmark stage of our long term studies of a protein called Pex11, which has an important role in the cellular processes linked to fats metabolism, but its molecular function has not been known until our study. We have now discovered that Pex11 senses acetyl-CoA, a central molecule in the cellular energy metabolism, and transfers information to the level of regulation of expression of some genes that are crucial for energy metabolism. In addition, we have found a small molecule agonist of Pex11 that can modulate the regulation of the expression of the respective genes. These results represent an important step in our understanding of cellular processes leading to obesity and type 2 diabetes, and provide a new avenue for finding medical treatment for these conditions.

Quantitative trait is a term that describes variability in the expression of a phenotypic trait that shows continuous variability, and is the net result of multiple genetic loci, called quantitative trait loci (QTLs). The majority of the economically important phenotypic traits in strains of industrial microorganisms are quantitative traits. In collaboration with our colleagues from the University of Graz, Austria, from the University of Toronto, Canada, and from the Faculty for Computer and Information Sciences of the University of Ljubljana, we have developed a method to identify QTLs in yeast at the level of a single allele, and the genetic interactions between them, in a quantitatively defined population of genetically different yeast strains. Subsequently, using established methods, transfer of identified alleles into a yeast strain with a different allele version is then performed to generate a biotechnologically improved strain. This method provides a step towards a new approach for a knowledge-based and targeted design of industrial microbiological strains (Figure 5).

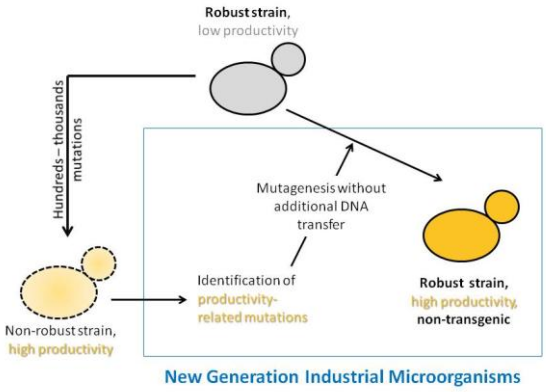

Figure 5. A method to design new generation strains of industrial microorganisms.

Evolutionary genomics and study of retrotransposons

Early evolutionary analyses of $sPLA_2$ toxins in venomous animals took place in the nineties of the last century, in the so called ”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 invited review (D. Kordiš, *Acta Chimica Slovenica*, 58 (2011), 638–646) we highlighted how the progress in the last decade has increased our understanding of the evolution of $sPLA_2$ toxins in venomous animals.

Genome-wide studies of intron dynamics in mammalian orthologous genes have found convincing evidence for loss of introns but very little for intron turnover. Similarly, large-scale analysis of intron dynamics in a few vertebrate genomes has identified only intron losses and no gains, indicating that intron gain is an extremely rare event in vertebrate evolution. These studies suggest that the intron-rich genomes of vertebrates do not allow intron gain. We searched for evidence of *de novo* intron gain in domesticated genes from an analysis of their exon/intron structures. A phylogenomic approach has been used to analyse all domesticated genes in mammals and chordates that originated from the coding regions of transposable elements (D. Kordiš, *Biology Direct*, 6-59 (2011)). Gain of introns in domesticated genes has been reconstructed on well established mammalian, vertebrate and chordate phylogenies, and examined as to where and when the gain events occurred. The locations, sizes and amounts of *de novo* introns gained in the domesticated genes during the evolution of mammals and chordates have been analyzed. A significant amount of intron gain was found only in domesticated genes of placental mammals, where more than 70 cases were identified. *De novo* gained introns show clear positional bias, since they are distributed mainly in the 5'-UTR and coding regions, while the 3'-UTR introns are very rare. In the coding regions of some domesticated genes up to 8 *de novo* gained introns have been found. Intron densities in Eutheria-specific domesticated genes and in older domesticated genes that originated early in vertebrates are lower than those in ”normal“ mammalian and vertebrate genes (Figure 6).

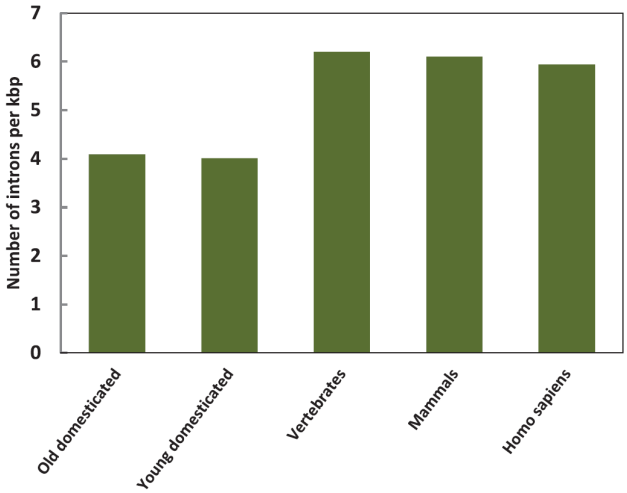

Figure 6. Intron densities in domesticated genes compared with vertebrate, mammalian and human ”normal“ genes. The figure is reproduced from D. Kordiš, *Biology Direct*, 6:59 (2011).

Surprisingly, the majority of intron gains have occurred in the ancestor of placentals. This study (D. Kordiš, *Biology Direct*, 6-59 (2011)) provides the first evidence for numerous intron gains in the ancestor of placental mammals and demonstrates that adequate taxon sampling is crucial

for reconstructing intron evolution. The findings of this comprehensive study challenge the current view on the evolutionary stasis in intron dynamics during the last 100–200 My. Domesticated genes could constitute an excellent system for the analysis of the mechanisms of intron gain in placental mammals.

Efts and adult specimens of the red-spotted newt *Notophthalmus viridescens* from various locations in Canada and USA were analyzed for the presence of tetrodotoxin (TTX) and of its analogues 6-epitetrodotoxin and 11-oxotetrodotoxin. Considerable individual variations in toxin levels were found within and among populations from New Hampshire, New York, Pennsylvania, and Virginia ranging from non-detectable to 69 µg TTX per gram of newt. TTX and its analogues were absent in efts and adults from various locations in the Canadian province Nova Scotia, the northernmost distribution 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. An exogenous source of TTX in the newts either via the food chain or by synthesis of symbiotic bacteria is suggested to explain the high variability and lack of TTX in certain populations. A paper about this topic will be published next year.

In 2011 we collaborated with our colleagues from the Faculty of Chemistry and Chemical Technology, University of Ljubljana (UL), also on a new basic research project on how APOBEC3 proteins inhibit L2 retrotransposon multiplication. Explanation of the mechanism of action of APOBEC3 proteins is very important as these proteins block proliferation of numerous retrotransposons and retroviruses, among them also HIV.

Other subjects

In 2011 we also collaborated at several projects out of the thematic scope of our department or the programme group ”Toxins and biomembranes“.

By analyzing the DNA and RNA samples from patients with unipolar depression we participated in the pharmacogenetic study of alternative antidepressant response of these people (K. Malki et al., *Biological Psychiatry*, 69 (2011), 360–365). With a structural analysis we participated in the identification of cysteine protease inhibitor from the filamentous yeast *Trichophyton mentagrophytes* on the project from the Veterinary Faculty, UL (B. Premrov Bajuk et al., *Acta Chimica Slovenica,* 58 (2011), 33–40). We also structurally characterized lectins isolated from the basidomycete *Clitocybe nebularis* isolated by the colleagues from the Department of Biochemistry, the Jožef Stefan Institute (J. Pohleven et al., *Applied Microbiology and Biotechnology*, 91 (2011), 1141–1148). In the case of amyotrophic lateral sclerosis and TDP-43 proteinopathies, the TDP-43 protein localizes and aggregates in the cytosol rather than in the cell nucleus. We collaborated in the description of action of TDP-43 on the RNA level (J.R. Tollervey et al., *Nature Neuroscience*, 14 (2011), 452–458). In collaboration with the Utrecht University NMR centre, we participated in the development of an original protocol for synthesis of a lanthanide tag for paramagnetic labelling of proteins in NMR experiments (F. Peters et al., *Journal of Biomolecular NMR*, 51 (2011), 329–337). With the same group we also prepared a chapter in the monography ”NMR of Biomolecules“ that will be published in 2012 by the Wiley publisher. In 2011 we also started the collaboration within the other two new basic research projects. With colleagues from the Biotechnical faculty, UL, we initiated a research on the apoptotic effects of alkylpyridinium compounds on lung adenocarcinoma cells and changes of membrane lipid structure in the pathological state.

Two worth mentioning achievements of the department in 2011 are connected with the late Professor Franc Gubenšek. First, to commemorate his memory we organized a special section at the congress of the European Section of the International Society on Toxinology in Valencia,

Spain. The section was very well attended and accepted. Second, we edited a special issue of the *Acta Chimica Slovenica* journal (Figure 7). In this issue some of the most prominent scientists in the field of toxinology contributed their papers.

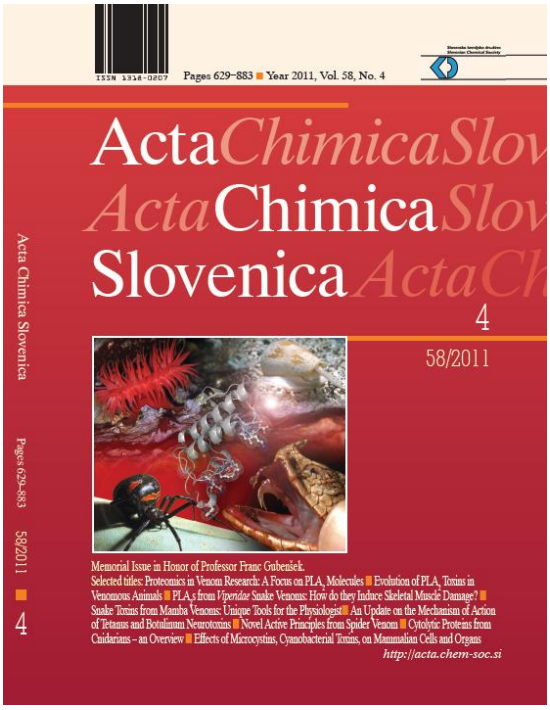

Figure 7. Cover page of the fourth issue of *Acta Chimica Slovenica* 2011, dedicated to the memory of the late Professor Franc Gubenšek, one of the founders of toxinology in Slovenia.