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 ($sPLA_2$)

The major research topic 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 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 year 2008 we studied the molecular mechanism of action of ammodytoxin (Atx), a presynaptically neurotoxic $sPLA_2$ from the long-nosed viper (*Vipera a. ammodytes*) venom, on murine cell lines and on murine and rat neuro-muscular (NM) preparations. Our results, obtained by fluorescent and electron microscopy (EM) analysis of a cell culture, have demonstrated the uptake of Atx into motoneuron-like cells, as well as its translocation into the cytosol (Figure 1).

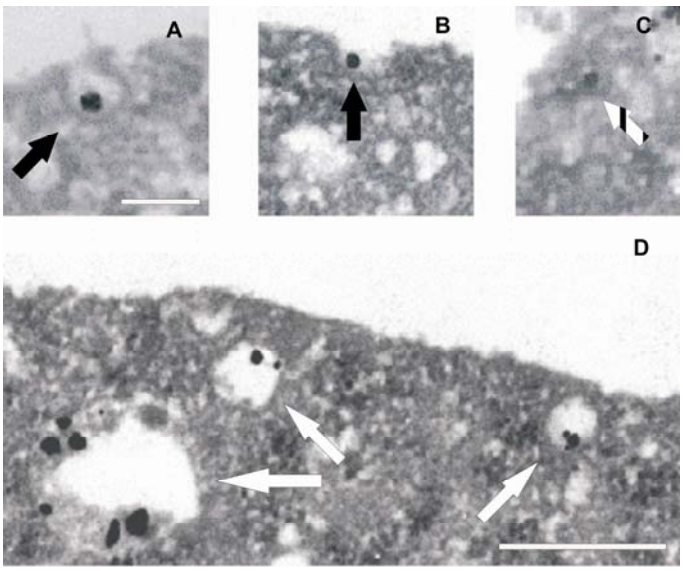

Figure 1. Localization of AtxA(N79C)-nanogold in motoneuron-like cells.

The results from microscopy studies were undoubtedly confirmed by detection of the formation of the complex between the toxin and cytosolic proteins, calmodulin (CaM) and 14-3-3, in living cells [COBISS.SI-ID 21453095]. In this way, we confirmed also *in vivo* the results obtained *in vitro*, that the cytosolic concentrations of Ca^{2+} allow the interaction between Atx and CaM [COBISS.SI-ID 21997607]. We were the first to demonstrate that an $sPLA_2$ can translocate into the cytosol of a nerve cell from the extracellular space. Exposure of the cells in culture to the toxic $sPLA_2$ *in vitro* resulted in the apoptotic death of cells [COBISS.SI-ID 24640729]. As the recent results show, one of the main factors of apoptotic cell death is a particular product of the enzymatic degradation of cellular membranes by the toxic $sPLA_2$. The photoreactive derivative of AtxC, sulfo-SBED-AtxC, was used to develop a new method for targeted therapy of cancer.

We synthesized the conjugate of sulfo-SBED-AtxC and “targeting” antibodies, which specifically recognized the CaCo-2 cancer cells. Following the specific binding on the cancer cells, the conjugate was internalized into the cells. In the reducing conditions of the cytosol the toxin molecule was released [COBISS.SI-ID 21313831].
Mitochondria are one of the main targets of Atx following its internalization into the nerve cells. The consequence of the enzymatic action of Atx on mitochondria in the nerve ending is also reduced production of ATP which is likely one of the main reasons for the interruption of the cycling of synaptic vesicles filled with neurotransmitter. The data gathered on the NM preparations were published in the distinguished medical pathophysiology journal [COBISS.SI-ID 21998375]. The high relevance of our report is reflected by its announcement on the cover page of the journal, presenting a collage of figures from the paper (Figure 2).

Figure 2. Cover page of the *Journal of Neuropathology and Experimental Neurology* with a collection of images from our paper.

Atx was labelled with a nanogold particle and used to block *in vivo* mouse NM junctions. Labelled junctions were isolated and in collaboration with the colleagues from the Newcastle University analysed by EM. We demonstrated the localization of the nanogold toxin derivative inside the nerve ending, which was done for the first time in the case of the nerve ending of a motoneuron, not only for a neurotoxic but for any $sPLA_2$. These results are in preparation for publication.
The study of the topology of the interaction of Atx with CaM, which is ready for submission, revealed two very interesting facts: the stability and the phospholipase activity of Atx increased substantially in the complex with CaM both in reducing (cytosol-like) and non-reducing conditions. We performed a detailed kinetic study of activation of the enzymatic activity of Atx and some other $sPLA_2$ with CaM. The most accurate description of the activation is by the non-essential activation model and the Atx-CaM system is the first practical example discovered. The results are in preparation for publication. Following the demonstration of the interaction of Atx with CaM in the cytosol of motoneuron-like cells [COBISS.SI-ID 21453095] and internalization of the toxin into the nerve ending of a motoneuron, this finding indicates the enzymatic activity of $sPLA_2$ also in the cytosol and nucleus of mammalian cells, its role in the process of ß-

neurotoxicity and in some other processes connected to endogenous $sPLA_2$ (*e.g.* at nuclear signalling, mRNA transport and apoptosis). With obtained results we further strengthened our hypothesis about the molecular mechanism of action of presynaptically neurotoxic $sPLA_2$, which claims that for the full expression of the toxic action of these $sPLA_2$s their internalization into the nerve cell is crucial. In order to exactly describe the happening at the nerve ending on the molecular level, *e.g.* molecular details of internalization of Atx into the cytosol of the nerve cell and its translocation into the mitochondria, we continued in this year with efforts to identify some known Atx-receptors: R25, R45 and R47, however, we have not come to final conclusions yet. As well, we were looking for novel Atx-binding molecules (proteins, lipids and glycolipids).
In the past year we published an extensive study on the structural features of presynaptically neurotoxic snake $sPLA_2$s that are responsible for their potent and specific action [COBISS.SI-ID 21585959]. To identify the residues that distinguish a highly neurotoxic ammodytoxin A from a structurally similar but more than two orders of magnitude less toxic Russell's viper (*Daboia r. russelli*) $sPLA_2$, VIIIa, we prepared a range of mutants and compared their properties. The results showed that the structural features that confer high neurotoxicity to AtxA extend from its C-terminal part, with a central role of the residues Y115, I116, R118, N119 (the YIRN cluster) and F124, across the interfacial binding surface (IBS) in the vicinity of F24, to the N-terminal helix whose residues M7 and G11 are located on the edges of the IBS. Binding studies indicated that the surface of interaction with the neuronal M-type $sPLA_2$ receptor extends over a similar region of the molecule. In addition, the YIRN cluster of AtxA is crucial for the high-affinity interaction with two intracellular binding proteins, cytosolic CaM and mitochondrial R25. The concept of a single "presynaptic neurotoxic site" on the surface of snake venom $sPLA_2$s is not consistent with these results which suggest that different parts of the toxin molecule are involved in distinct steps of presynaptic neurotoxicity (Figure 3).

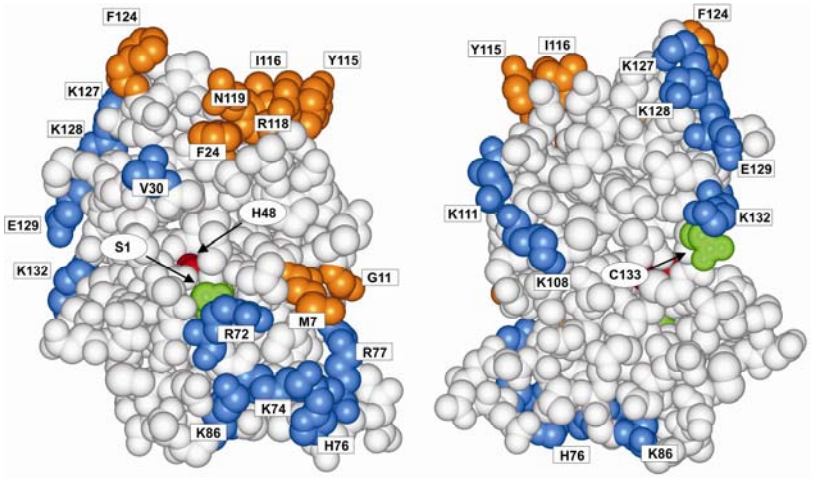

Figure 3. Structural model of AtxA showing the residues involved in ß-neurotoxicity. It appears that different parts of the toxin molecule are involved in different stages of the complex process of ß-neurotoxicity. Consequently, it is not appropriate to use the term ''a distinct presynaptic neurotoxic site'' on the toxin molecule.

We participated in the development of the chromatographic method for the rapid and accurate determination of Atx content in venoms of *Vipera a. ammodytes* specimens [COBISS.SI-ID 21233959]. Namely, we found a high correlation between the content of Atx in the venom and the suitability of the venom for the preparation of high quality antiserum by animal immunization [COBISS.SI-ID 21825831]. These findings will substantially lower the price of the high quality antiserum production and reduce the work on animals in the process of its preparation.

In the beginning of this year, we published a review article on pathophysiological role of $sPLA_2$s where, among others, their involvement in various cancer and neurodegenerative disorders has been presented [COBISS.SI-ID 21552167]. In 2008, we started a new project aimed at exploring the role of $sPLA_2$s in the development and progression of breast cancer [J3-0386]. Currently, ten structurally distinct $sPLA_2$ enzymes are known in humans, which differ in enzymatic activity, membrane binding and receptor binding affinities. These enzymes show diverse tissue expression patterns, suggesting tissue-specific pathophysiological roles and mechanisms of action. Our hypothesis is that certain $sPLA_2$ enzymes are involved in cellular processes that have an impact on breast cancer development and progression. In the first year of the project we have focused our attention on identifying members of the $sPLA_2$ family that display differential levels of expression in cell models of breast cancer. We used several human breast cancer cell lines, which differ in their invasiveness *in vitro*, tumourigenicity *in vivo* and steroid hormone receptor status, as well as two nontumourigenic cell lines, which retain the characteristics of normal breast epithelial cells. We determined the differences in mRNA expression levels of the whole set of human $sPLA_2$s in the selected cell models using real-time quantitative PCR (qPCR). Our results indicate that breast cancer cell lines with different tumourigenic characteristics, reflecting the progression of the disease from less aggressive and hormone-dependent to the more invasive and hormone-independent forms, display different levels of expression of particular $sPLA_2$s.
Investigation has also been focused on the presence and role of endogenous $sPLA_2$s in the (peripheral) nervous system, which is also an important target site of action of snake venom neurotoxic $sPLA_2$s. In collaboration with the Institute of Pathophysiology, Medical Faculty, University of Ljubljana, we analyzed a co-culture of rat embryonal spinal cord explants and human skeletal muscle cells (Figure 4) by immunocytochemistry for the presence of five groups of endogenous $sPLA_2$s, IB, IIA, IIE, V and X.

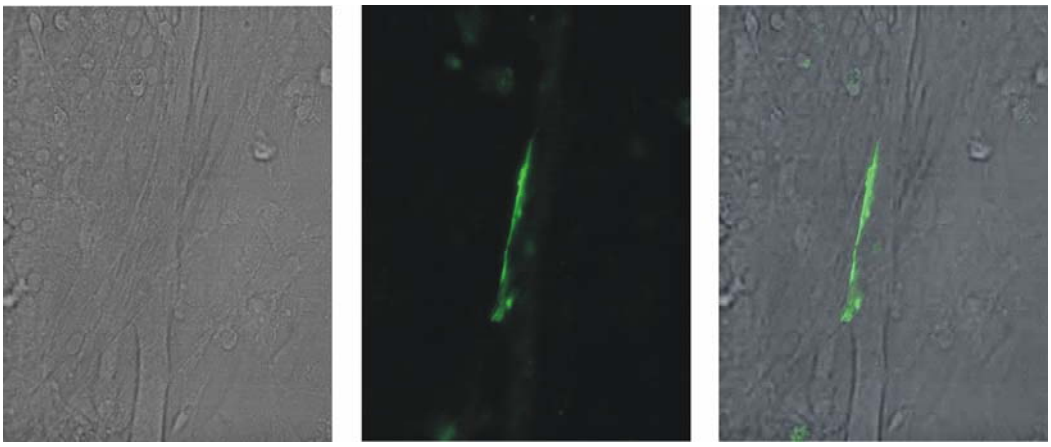

Figure 4. Heterologous co-culture of the explant from rat embryo spinal cord and human skeletal muscles as a model for the study of activity of endogenous and snake venom $sPLA_2$s at the neuromuscular junction. The heterologous junction (synapse) is stained green. Accomplished in collaboration with the Institute of Pathophysiology, Medical Faculty, University of Ljubljana.

This heterologous co-culture could also serve as a model for the study of action of endogenous and snake $sPLA_2$s in the region of neuromuscular junction. Preliminary results showed the presence of group IIA, V and X $sPLA_2$s in different cell types in the co-culture (neurons, their support cells, muscle cells). The demonstration of different $sPLA_2$s in the co-culture, able to form functional neuromuscular junctions, has provided a good basis for the investigation of $sPLA_2$s in

less complex systems of neuronal cells. We confirmed the presence of group X $sPLA_2$ in a mouse motoneuronal cell line, and groups V and X in a rat neuron-like cell line. Aiming to investigate the specificity of immunodetection of different groups of $sPLA_2$s, we are currently occupied with the production of certain recombinant mammalian $sPLA_2$s. The latter will be used to test the potential cross-reactivity of particular antibodies and to observe the effect of different $sPLA_2$s on the neuronal cells and functional heterologous co-culture.

Other pharmacologically active components from natural toxins

We studied *Vipera a. ammodytes* venom components that affect haemostasis, particularly different proteases, and published the work describing the isolation and characterization of two novel coagulation factor X activators with potential to treat patients with dysfunctional factors IXa or VIIa [COBISS.SI-ID 21894439]. In this year we started also with a new project dedicated to the development of a potential of fibrinogenolytic and non-haemorrhagic ammodytase to serve in the therapy of thrombosis [J3-0389].

In 2008 we continued the work on the EU 6FP integrated project "Conco". As one of the 20 partners we have been involved in the analysis of the genome, transcriptome and venom proteome of the marine snail *Conus consors*. We investigated the toxicity of the Cc001 conopeptide from the venom of this snail possessing a potent inhibitory activity on Na^+ voltage-gated channels. No cytotoxicity on neuroblastoma cells was observed even at a 100 μM concentration, whereas a transient cytotoxicity was detected in myoblasts and motoneuron-like cells. Currently, we are analyzing different HPLC fractions of the *C. consors* venom for the presence of phospholipase activity. Venoms of two species of cone snails, piscivourous *C. consors* (whose prey is mainly fish) and vermivorous *C. quercinus* (whose prey are marine worms), have been successfully separated by 2D electrophoresis into individual protein components.

The methodology of preparation of the photoreactive derivative of AtxC was successfully used to prepare photoreactive derivatives of human SMOC ("secreted modular Ca^{2+} binding") proteins to study interactions with proteins in human serum [COBISS.SI-ID 21935399]. In collaboration with the group from the New York University Langone School of Medicine from New York we investigated the perturbation of transforming growth factor (TGF)-ss1 association with latent TGF-ß binding protein and found positive effects on inflammation and tumourigenesis [COBISS.SI-ID 22221351].

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

In 2008 we have made some important steps toward measuring yeast colony volumes on agar plates, which is an important advancement in high-throughput genetics (Figure 5). We have demonstrated that by application of this development into our experimental platform, the quality of generated data is significantly improved.

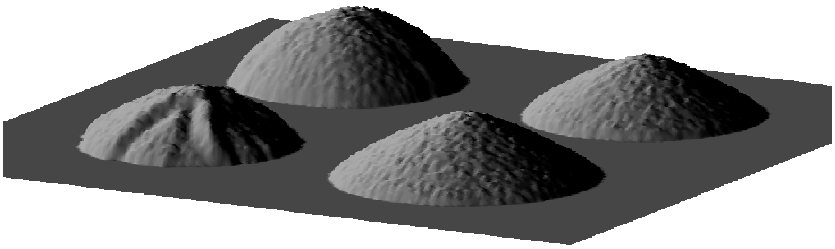

Figure 5. Reconstruction of yeast colonies shape on agar plate, which allows accurate determination of their volume.

Based on the interpretation of our high-throughput genetics data, we have elucidated the first contours of the core genetic network of membrane biology: we discovered a functional interaction between peroxisome proliferation and endocytosis and some of the genes/proteins involved in this pathway.
In close collaboration with the faculty of Computer and Information Sciences of the University of Ljubljana, we have started to analyze the not-yet-published 3rd generation data on the genetic interactome of yeast *Saccharomyces cerevisiae*, generated at the University of Toronto. This project is making a huge contribution towards the understanding and treatment of polygenetic diseases.
In collaboration with the University of Nova Gorica we have identified some novel targets of action of pesticides, which could explain some of their side effects and enable development of safer new compounds.
In the past year we have also initiated the work on a new applicative project. With our partners, Lek-Novartis pharmaceutical company and faculty of Computer and Information Sciences of the University of Ljubljana, we have started to develop novel knowledge technology approaches in drug discovery that are based on analysis and experiment planning in high-throughput genetics [L2-1112].

Evolutionary genomics of transposable elements and functional studies of retrotransposons

The origin of the novel mammalian genes (neogenes) from retroelement remains (e.g. gag and integrase) and their evolution has been until now only partially elucidated, due to the absence of the genome data or the limited analysis of a single family of neogenes. By using phylogenomic analysis (combining phylogenetic tree construction, integration of experimental data and differentiation of orthologs and paralogs) we obtained and characterized retroelement-derived neogenes from all currently available mammalian genomes (more than 50 different species available at NCBI and ENSEMBL) and their progenitors from the genomes of the key tetrapod genomes (amphibians and reptiles). Phylogenomic analysis provided very large amount of information for each novel neogene, such as genome sequence, gene structure, genome locus, chromosomal location, protein sequence, coding and non-coding regions as well as regulatory regions. With the analysis of numerous mammalian genomes (from monotremes, marsupials and four placental superoders) and by analysis of novel neogene families, an in depth insight into the origins, evolution, regulatory and functional diversification of diverse retroelement-derived neogenes in mammals has been obtained. We explained where and when domestication of retroelements occurred and how similar to the modern neogenes the first mammalian retroelement-derived neogenes were. Until now the evolutionary relationships of currently known retroelement-derived neogenes were not well resolved due to the poor taxonomic sampling. Novel data that we obtained from the genomes of monotremes, marsupials and basal placental superoders has greatly improved and finally resolved the evolutionary relationships of diverse neogenes. The well resolved evolutionary relationships of mammalian retroelement-derived neogenes are crucial for elucidation of their unusual dynamics of chromosomal mobility as well as for the timing of domestication.

Several APOBEC3 proteins (A3F and A3G), a group of cytidine deaminases restrict human immunodeficiency virus (HIV) replication in the absence of the viral infectivity factor (Vif) protein. However, Vif leads to their degradation and counteracts their effects. Another member, A3A, restricts some retrotransposons and another virus but not HIV. We reasoned that this failure was due to the lack of appropriate targeting. Thus, we fused A3A to another viral protein, Vpr, which binds p6 in Gag and is incorporated into viral cores. Indeed, the Vpr.A3A chimera but not A3A was found abundantly in the viral core. It also potently restricted the replication of HIV and

simian immunodeficiency virus (SIV) in the presence and absence of Vif. Since a high frequency of G to A mutations in viral cDNAs was identified, we assumed this antiviral activity was mediated by DNA editing. Interestingly, our fusion protein did not restrict murine leukemia virus, which does not incorporate Vpr. Thus, by appropriately targeting a potent single domain cytidine deaminase, we rendered HIV and SIV restriction resistant to Vif. Because the Vpr.A3A chimera inhibited SIV, such therapeutic strategies could be tried first in the monkey model of AIDS in rhesus macaques [COBISS.SI-ID 21302823].