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 (patho)physiology 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), both those from animal toxins and those endogenous to humans. We are interested in the molecular mechanisms of action of toxic $sPLA_2$s, particularly those with presynaptic neurotoxicity, anticoagulant activity and myotoxicity, as well as in the roles of endogenous $sPLA_2$s in pathological and physiological processes in mammals.

One of the characteristic pathologic effects of the neurotoxic $sPLA_2$ is their damage of mitochondria. Following the discovery of endogenous $sPLA_2$ in mitochondria of neuronal cells and colocalization of the fluorescently labelled ammodytoxin A (AtxA), a model neurotoxic $sPLA_2$ from the venom of the long-nosed viper (*Vipera a. ammodytes*), with mitochondria in the PC12 cell line, we focused in the past year on characterization of the influence of AtxA on mitochondria in this cell line. We followed the formation of free radicals and changes of mitochondrial membrane potential in differentiated and nondifferentiated PC12 cells after they were exposed to AtxA or some other $sPLA_2$. We measured these parameters also on isolated mitochondria. Some of the studies were performed in the scope of the bilateral project in collaboration with the University of Perugia.

In the past year we arranged and analyzed the results of the study of molecular mechanism of action of AtxA on mouse and rat neuro-muscular preparations *ex vivo* (Figure 1), obtained in the trilateral NATO Collaborative Linkage Grant collaboration with the Newcastle University and University of Strathclyde in Glasgow.

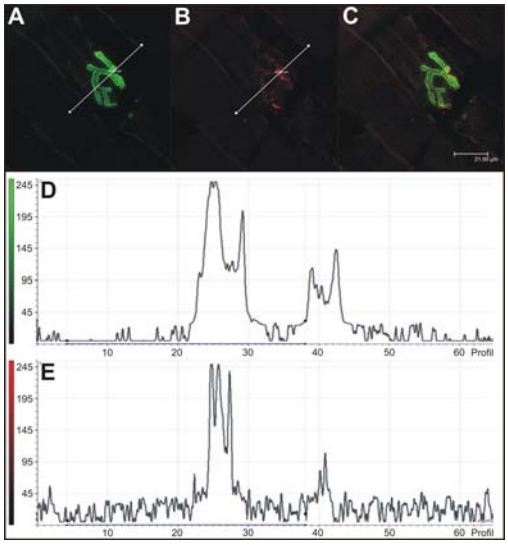

Figure 1. Longitudinal section of a mouse soleus muscle exposed to Alexa^{546}-conjugated AtxA revealed a perisynaptic localization of the toxin derivative. Red fluorescence signal belongs to Alexa-conjugated toxin while AChR (postsynaptic localization) were counter-labelled with green fluorescence using FITC-conjugated α-bungarotoxin.

One of the most important riddles to solve to reveal the molecular basis of action of presynaptically toxic $sPLA_2$ is to structurally identity their specific receptor on the presynaptic membrane of a motoneuron. Using the radioactive derivative of AtxC, a natural isoform of AtxA, the N-type receptors, tentatively the key receptors for expressing the neurotoxicity of $sPLA_2$, could not been detected in the rat brain. However, similar receptors have been found in the electric organ (modified peripheral nerve system) of *Torpedo marmorata* and we started to develop the isolation procedure from this tissue. We looked for specific Atx receptors also in lipid rafts but until now we have not found any new receptors. It is known that receptors for natural neurotoxins are not exclusively of a protein nature. They can be also glycolipids. From presynaptic membranes of the porcine cerebral cortex we isolated the glycolipid fraction and evaluated the interaction of Atx with this fraction. The interaction of Atx with glycolipids was studied also on artificial vesicles prepared from the pure (glyco)lipid components in known ratios. In collaboration with the Institute of Biochemistry from Medical faculty, University of Ljubljana, we continued with the study of the influence of Atx on the G-protein-coupled receptors in rat brain neurons. At present, the activation of G-proteins can be neither confirmed nor neglected.
To advance the study of $sPLA_2$, the development of new molecular tools is necessary. For this purpose we constructed an original photo-reactive derivative of AtxC, sulfo-SBED-AtxC. Its preparation, characterization and utility we described in the paper [COBISS.SI-ID 20950823]. Using this derivative we discovered two novel neuronal binding proteins for Atx, developed more effective procedure to isolate R25, which is the Atx receptor in neuronal mitochondria, and studied the topology of interactions between Atx and its known binding proteins, calmodulin (CaM), protein disulphide isomerase, 14-3-3 proteins and activated blood coagulation factor X (FXa). Based on these studies, we built a model of the Atx–CaM complex (Figure 2).

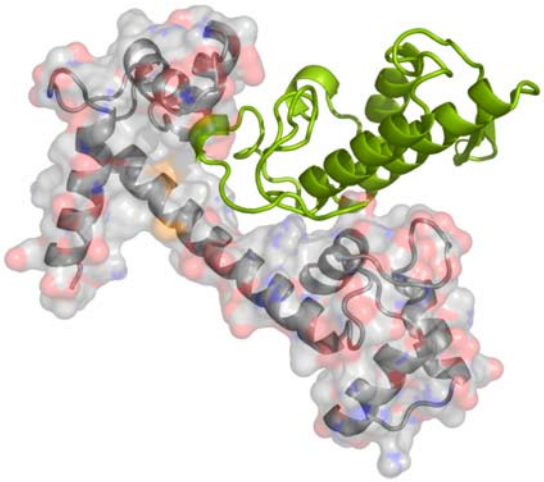

Figure 2. The tridimensional model of the complex between AtxA (green) and CaM.

On a mouse motoneuronal cell line and using sulfo-SBED-AtxC we unambiguously demonstrated for the first time that the $sPLA_2$ was translocated into the cytosol of a eukaryotic cell (publication submitted). We demonstrated that the internalization of Atx into neuronal cells is highly dependent on the presence of calcium (Ca^{2+}) ions in the extracellular space. In the absence of Ca^{2+}, the neurotoxin was still internalized, but to a much lesser extent. Ca^{2+} ions are important for enzymatic activity of Atx, which is essential for its neurotoxicity. After the internalization of Atx into the model mouse motoneurons, we observed a significant reduction in the staining intensity for two synaptic vesicle proteins, synaptophysin and synaptotagmin, as well as a reorganization of the F-actin cytoskeleton. Atx also induced cell death of motoneurons,

likely through the mitochondrial pathway, which we still have to investigate in details. By using fluorescently labelled Atx and antibodies to clathrin, we showed that Atx internalized largely by clathrin-mediated endocytosis, although other pathways, such as through synaptic vesicles, could not be excluded. The toxin uptake was efficiently suppressed by the addition of a nontoxic and enzymatically active $sPLA_2$, ammodytin I_2, indicating the presence of specific receptors on the surface of motoneuronal cells involved in the internalization. Based on the results obtained, we conclude that Atx, following the internalization into the neuronal cell (Figure 3), undergoes intracellular trafficking to different organelles and to some extent also into the cytosol. Until now, we have not been able to clearly demonstrate the presence of similar or identical receptors on the presynaptic membrane of the neuro-muscular junction of the victim of a snake bite and on the model motoneurons.

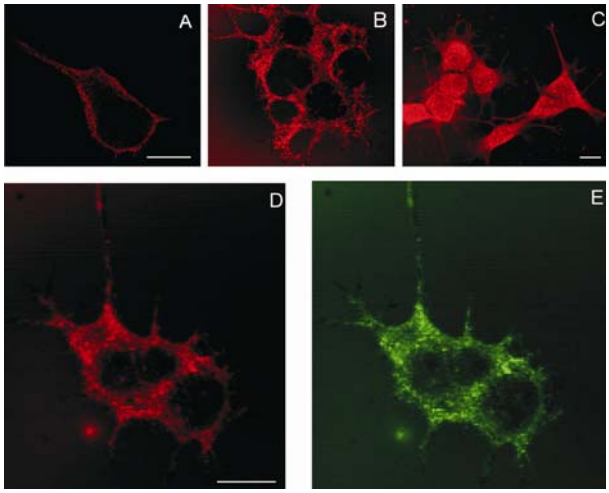

Figure 3. Time course of the internalization of fluorescently labelled AtxA(N79C)-Texas Red into the cells of a motoneuronal line (A, after 2 min; B, D and E, after 15 min; C, after 60 min; E, fluorescently labelled AtxA in the cell, additionally stained by specific antibodies to AtxA (green)).

Aiming to develop a method for targeted therapy of cancer, we prepared the conjugate between sulfo-SBED-AtxC and "targeting" antibodies which specifically recognized CaCo-2 cancer cells and internalized the toxin into these cells [COBISS.SI-ID 21313831].
Collaborating in the bilateral project, we succeeded together with our colleagues from the Institute Pasteur in Paris to crystallize AtxA, its N-terminal fusion mutant and AtxC and determine their tridimensional structures. In the past year we also continued to search for conditions to crystallize AtxA in the complex with FXa. The purpose of these studies is to develop innovative anticoagulant drugs on the basis of the structure of AtxA which is interacting with FXa. We also continued with the cocrystallization of AtxA and its cytosolic target protein CaM. We proved that concentrations of Ca^{2+} present in the cytosol of eukaryotic cells support the interaction between Atx and CaM (publication submitted), an additional argument in favour of our hypothesis about the intracellular action of presynaptically neurotoxic $sPLA_2$, which we described in detail in the invited review paper [COBISS.SI-ID 21173543]. Related to this hypothesis, the observation that the enzymatic activity of Atx increases substantially when it is in the complex with CaM is very interesting. Kinetic studies of the activation of enzyme activity of Atx and some other $sPLA_2$ in the presence of CaM are underway.
In this year, we concluded an extensive study on the action of enzymatically inactive myotoxic phospholipases using ammodytin L (AtnL), a group IIA $sPLA_2$ homologue, as a model

[COBISS.SI-ID 21167399]. The characteristic of these myotoxins is that the Asp-49 residue in the so called “calcium binding loop” is usually substituted with a Lys or rarely with a Ser residue. AtnL is one of the two known Ser-49 homologues. In addition to this replacement, several other substitutions can be found in the molecules of enzymatically inactive snake $sPLA_2$s in the region of the Ca^{2+} binding loop which is involved in the coordinative binding of a cofactor, Ca^{2+} ion, essential for the catalytic activity of $sPLA_2$ enzymes. By site-directed mutagenesis, we prepared two enzymatically active quaternary mutants of AtnL (H28Y/L31V,W/N33G/S49D), differing at position 31. The LV-mutant possessed Val while the LW-mutant had Trp at this place. Both mutants, in contrast to recombinant wild type AtnL, efficiently hydrolyzed phospholipid vesicles of different compositions, as expected, LW-mutant being approximately 50-fold more active than the LV-mutant. In contrast to AtnL but similarly to AtxA, a homologous neurotoxic $sPLA_2$, both mutants exhibited enzyme activity-dependent membrane-damage. However, both mutants also exhibited the potent Ca^{2+}-independent disruption of vesicle integrity the characteristic of AtnL, but not of AtxA. Although the LV and, especially, the LW-mutant display higher cytotoxicity and higher lethal potency, they have a lower Ca^{2+}-independent membrane-damaging potency and reduced specificity in targeting muscle fibres *in vitro* than AtnL. Our results indicate that, during the evolution, Lys-49 and Ser-49 $sPLA_2$ myotoxins have lost their Ca^{2+}-binding ability and enzymatic activity through subtle changes in the Ca^{2+}-binding network. At the same time, the rest of the catalytic machinery has not been affected, thereby optimizing their Ca^{2+}-independent membrane-damaging ability and myotoxic activity.

In the scope of the bilateral project with the Institute of Immunology in Zagreb and in collaboration with the Bia Separations company from Ljubljana we developed the rapid chromatographic method for accurate determination of Atx content in venoms of *Vipera a. ammodytes* specimens [COBISS.SI-ID 21233959]. A high correlation has been namely found between the content of Atx in the venom and the suitability of the venom for the preparation of high quality antiserum by animal immunization. Our findings will lower the price of the high quality antiserum production and reduce the work on animals in the process of their preparation.

Studying the role of endogenous $sPLA_2$ in physiological and pathological processes in mammals we designed oligonucleotide primers and optimized PCR conditions for amplification of mRNA of 4 enzymatically most active $sPLA_2$ groups in mice, rats and humans. In addition, we prepared several plasmid constructs to investigate the roles of human endogenous $sPLA_2$s in cancer diseases and began with the culturing of cell lines of breast cancer origin.

The research on $sPLA_2$ inhibitors is conveyed in the first place aiming to discover new molecules to control the activity of endogenous as well as exogenous $sPLA_2$. In year 2007 we finalized the characterization of the $sPLA_2$ inhibitor from the serum of *Vipera a. ammodytes* and published the results [COBISS.SI-ID 21233703].

Other pharmacologically active components from natural toxins

In 2007 we continued with the research of the *Vipera a. ammodytes* venom components that affect hemostasis. We especially focused on biochemical and pharmacological characterization of hemorrhagic and nonhemorrhagic metalloproteinases (MP). The description of fibrinogenolytic ammodytase with a high applicative potential for the therapy of thromboses was published [COBISS.SI-ID 20706855]. We also prepared an application of the project which goal is to develop the ammodytase towards its medical use. We tested the influence of this snake venom MP on epithelial cells. With the development of the specific antibodies towards the most toxic components of the *Vipera a. ammodytes* venom we continued, together with the Institute of Immunology in Zagreb, the work on preparation of safer antisera for treatment of envenomed patients. Besides neurotoxic Atx these are also hemorrhagic MP. The produced antibodies have been successfully used also in purification procedures as well as at further characterization of these venom components.

In this year the work on the integrated EC-FP-6 project "Conco" started. As one of the 20 partners we have been involved in the analysis of genome and venom proteome of the venomous fish-hunting cone snail *Conus consors*, preparation of the synthetic polypeptide venom library and pharmacological screening aiming at discovering drug leads to develop new biological drugs.

Phenomics in yeast *Saccharomyces cerevisiae*

We have developed an experimental method and underlying bioinformatics tools for quantitative determination of yeast strains' growth rates with agar-based assay, which enables high-throughput chemical genomics analysis. In combination with genetic interaction data, this approach, named "context-dependent genetic interaction analysis", enables identification of drug targets, mechanisms of action of small molecules or proteins, and gene function (Figure 4).

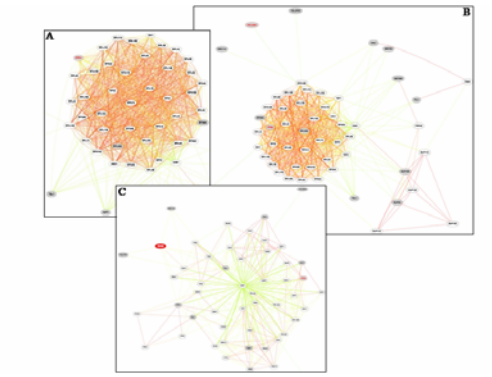

Figure 4. Context-dependent genetic interaction analysis. Three networks of interactions between functionally related yeast genes, determined through genetic interactions with a studied gene, and identifying the function of the studied gene under three different conditions (A, B and C, respectively).

Using this approach we have been studying cellular responses to different perturbations of membranes of lipid metabolism. We have started identifying the genes that build a core network which regulates lipid and membrane homeostasis in eukaryotic cells.

In collaboration with the University of Graz, Austria, we have developed a high-throughput method for analysis of organelle biology (biosynthesis, inheritance, proliferation and degradation) in the context of all yeast single-gene deletion yeast strains (Figure 5).

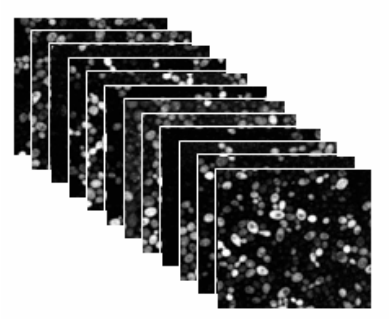

Figure 5. Images of 12 identified strains, out of total 4800 tested, with aberrant peroxisome biogenesis.

We have rigorously shown that combination of gene expression and genetic interaction data can very accurately predict the mechanism of action of pharmacologically active substances (publication submitted). In collaboration with the faculty of Computer and Information Sciences of the University of Ljubljana, we have developed a computational method for the implementation of this discovery (Figure 6).

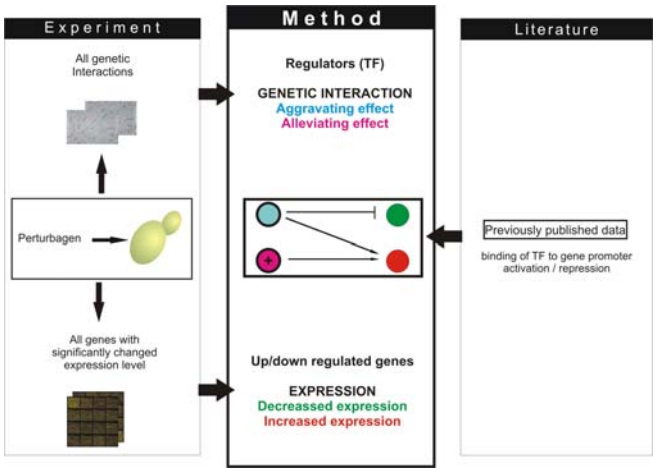

Figure 6. Schematic representation of the method for prediction of the mechanism of action of pharmacologically active substances based on gene expression and genetic interaction data. Experimental data on the effects of the perturbagen on the transcriptome and on its genetic interactome, together with literature data on the nature of the effect of the regulators in genetic interaction with genes with a significantly changed expression level, are used as input data. The method generates a wiring diagram of a hypothetical model of the molecular mechanism of the action of the perturbagen.

In collaboration with the University of Pavia we have developed a methodology for learning gene regulatory networks from DNA microarray data based on the integration of different data and knowledge sources. We applied the method to *S. cerevisiae* experiments, focusing our attention on cell cycle regulatory mechanisms, and biologically evaluated it on known cell cycle genes against independent knowledge sources [COBISS.SI-ID 21202727].

Evolutionary genomics of transposable elements and functional studies of retrotransposons

We continued our research on the eukaryotic transposable elements. Analyses of diverse eukaryotic genome databases provided the answers on numerous interesting questions. Genome architectures of mammals and birds is quite unusual, since they have lost diversity of transposable elements, but the remaining transposable elements reached very high copy numbers and have reshaped these genomes. Until now it was not known when and why such huge changes occurred. The answers are hidden in the genomes of land vertebrates and especially of the reptiles. In the genomes of the lizard (*Anolis carolinensis*), crocodiles and turtles we analyzed numerous retroelements and DNA transposons. Very large retroelement diversity was discovered in the lizard genome only, but not in the genomes of crocodiles and turtles. With the help of the palaeogenomic analysis of transposable elements in diverse reptilian groups and planetary-biological approach we found the answer on the long-standing question why are mammalian and avian genomes so different and unique among the metazoans. In the genome of the lizard (*Anolis carolinensis*) we analyzed the L1 retrotransposons, and discovered enormous L1 diversity, that is currently the highest among the vertebrates. We found more than

150 diverse L1 families; the copy numbers of L1s per family are very variable. This discovery is very important, since it provides the direct evidence that L1 repertoire in the genome of the mammalian ancestor was also very rich. Fungi, our closest opisthokont relatives, and the metazoans possess very different transposable element contents. Until now it was not explained when and how the metazoa-specific transposable element groups originated. We analyzed the transposable elements in the genomes of the most ancestral metazoan lineages, in sponges and cnidarians. We found that majority of the metazoa-specific transposable element groups originated very early in the last common ancestor of Metazoa and that some smaller transposable element lineages originated later. These analyses provided also the evidence for the enormous diversity of retroelements and DNA transposons in the genomes of sponges and cnidarians that is much higher than in vertebrates. The analyses of DNA transposons and retroelements in the key eukaryotic lineages provided the answer on the crucial question about the origin and evolution of DNA transposons and retroelements in eukaryotes. By the analysis of more than 300 eukaryotic genomes we provide the evidence where and when did particular superfamilies of DNA transposons and retroelements originated and also the mechanisms of their evolution. We studied the horizontal gene transfer in mammals, since we are the pioneers in this field, and reported in 1995 first such example [COBISS.SI-ID 10623015]. The availability of the very large number of mammalian genomes from all three major mammalian lineages has enabled the study of the horizontal gene transfer in mammals. We found that horizontal transfer is not limited on the retroelements, and we found a number of the new examples of the retroelement horizontal transfers between the insects and their predators (insectivores and bats). During the evolution of chromoviruses we found unusual Metaviridae with unique targeting domains (e.g. PHD domain) and additional ORFs (envelope proteins). The availability of the numerous genomes of the unicellular and basal eukaryotes enabled the analysis of the origin and evolution of the PHD domain and envelope proteins in eukaryotic Metaviridae. We studied also the interactions among the proteins, involved in RNA metabolism (P bodies) and the proteins of the LTR (HERV-K) and non-LTR retrotransposons (LINE-1). The colocalization of the proteins, encoded by the LTR and non-LTR retrotransposons, and the APOBEC3 proteins was studied in mammalian cells.