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.

Within the scope of our research on the molecular mechanism of action of presynaptically neurotoxic snake venom $sPLA_2$s, we became the first group in the world to successfully confirm the internalization of a presynaptically neurotoxic $sPLA_2$, ammodytoxin (Atx), from the venom of the long-nosed viper *Vipera ammodytes ammodytes*, in the motor nerve terminal *in vivo* (U. Logonder et al*., Experimental neurology*, 219 (2009), 591–596). In this study, we labelled a recombinant mutant of AtxA, AtxA(N79C), by covalently attaching nanogold particles and inoculated the conjugate into the limb of laboratory mice. Several hours after inoculation of the labelled toxin the soleus muscle was isolated, fixed in glutaraldehyde, exposed to a silver enhancing medium and ultra thin sections were prepared for transmission electron microscopy analysis. The results showed extensive mitochondrial damage and depletion of synaptic vesicles in most of the terminal boutons, which are typical indicators of neurotoxicity following exposure to neurotoxic $sPLA_2$s. Silver-enhanced particles were found concentrated in the perisynaptic area (particularly within the synaptic gutter, Schwann cell lamellae and mastocytes) and were taken up into the cytoplasm of the terminal boutons of the motor axon, where they were localized in mitochondrial and vesicle structures as well as in the cytosol (Figure 1).

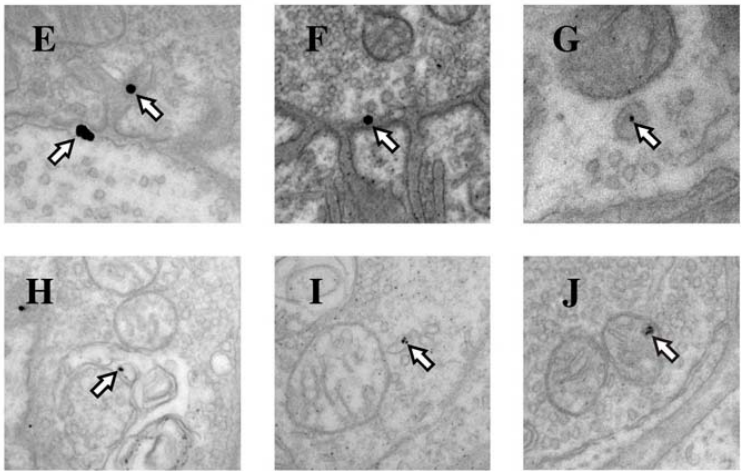

Figure 1. Electron micrographs of a terminal bouton from muscle fibres exposed to a mutant form of AtxA conjugated to nanogold particles and silver enhanced. High resolution images show the localization of particles to the plasma membrane of the terminal bouton (E and F) and internalized into the terminal bouton. Internalized particles are visible within vesicular structures (G and H), in the cytosol and mitochondria (I and J). The figure is reproduced from U. Logonder et al*., Experimental neurology*, 219 (2009), 591–596.

Detailed analysis of the influence of calmodulin (CaM), a cytosolic protein with a high affinity for AtxA, on the enzymatic activity of AtxA showed that CaM completely stabilized the enzyme/toxin in the reducing cytosol-like conditions and substantially increased its enzymatic activity in such conditions as well as in the non-reducing environment (L. Kovačič et al*., Biochemistry*, 48 (2009), 11319–11328). We concluded that CaM augmented the phospholipase activity of the $sPLA_2$ as a nonessential activator and suggested a model of structural-mechanistic relationships of the CaM-Atx interactions (Figure 2). In the same way, CaM activated also mammalian group V and X $sPLA_2$s. Our results provide further insight into the neurotoxic action of Atx and the mechanisms involved in the regulation of $sPLA_2$ activity within the cytosol.

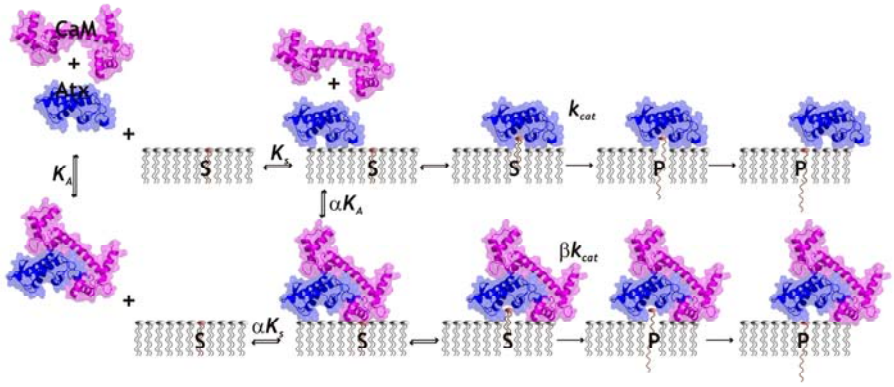

Figure 2. Structural-mechanistic relationships of the interaction between CaM and Atx. On binding to Atx, CaM modulates the association of Atx with the lipid membrane and its catalytic step. The figure is reproduced from L. Kovačič et al*., Biochemistry*, 48 (2009), 11319–11328.

Employing our *in vitro* model of mouse motoneuronal cells, we have observed that the neurotoxic AtxA causes a rapid dissipation of mitochondrial membrane potential (Figure 3) leading to cell death.

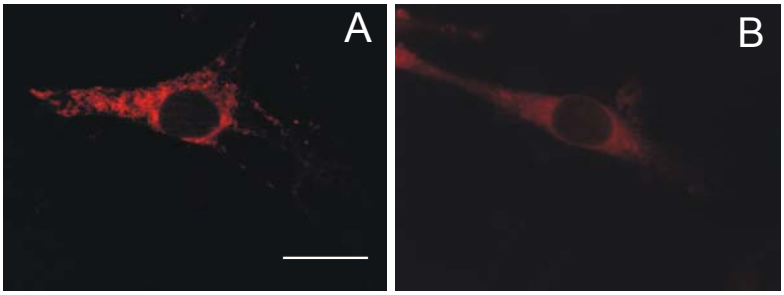

Figure 3. Fluorescence microscopy analysis of mitochondrial integrity in motoneuron-like cells without exposure to AtxA (A) and following the AtxA treatment (B). Weakening of the red fluorescence, characteristic for the healthy mitochondria, in the presence of AtxA indicates a fall of the mitochondrial membrane potential. The scale bar represents 20 μm. The figure is reproduced from Z. Jenko Pražnikar et al., *Annals of the New York academy of sciences*, 1152 (2009), 215–224.

The cellular changes accompanying the process, including activation of caspase 3 and structural changes in the plasma membrane, led us to conclude that the observed cell death is apoptotic (Z. Jenko Pražnikar et al., *Annals of the New York academy of sciences*, 1152 (2009), 215–224).

In our search for new compounds with potential inhibitory activity against AtxC, a natural isoform of AtxA, and pancreatic $sPLA_2$, we screened two phage-displayed random peptide libraries and found several short peptides expressing a binding affinity for both $sPLA_2$s, from groups IB and IIA. None of these peptides was, however, able to inhibit the enzymatic activity of the $sPLA_2$s efficiently *in vitro* (D. Gaser et al., *Acta chimica Slovenica*, 56 (2009), 712–717).

In collaboration with colleagues from the Pasteur Institute in Paris, crystal structures of AtxA and AtxC were determined (F.A. Saul et al., *Journal of structural biology*, 2009, doi:10.1016/j.jsb.2009.10.010). The two natural isoforms differ in only two amino acid residues in their C-terminal parts: while AtxA has Phe and Lys in positions 124 and 128, respectively, AtxC has Ile and Glu. However, in spite of minimal differences in their primary structures these two $sPLA_2$s differ substantially in their enzymatic activity, neurotoxicity and anticoagulant activity. The tridimensional structures of AtxA and AtxC revealed that the amino acid variation in position 128 causes significant local structural alterations between the two proteins. The result is weaker interaction of AtxC with blood coagulation factor X than that of AtxA and consequently a lower anticoagulant activity of AtxC comparing to AtxA. On the contrary, the amino acid residue variation in position 124 does not induce evident conformational change. AtxA is thus more toxic than AtxC because of the specific nature of Phe that it has in this position (Figure 4).

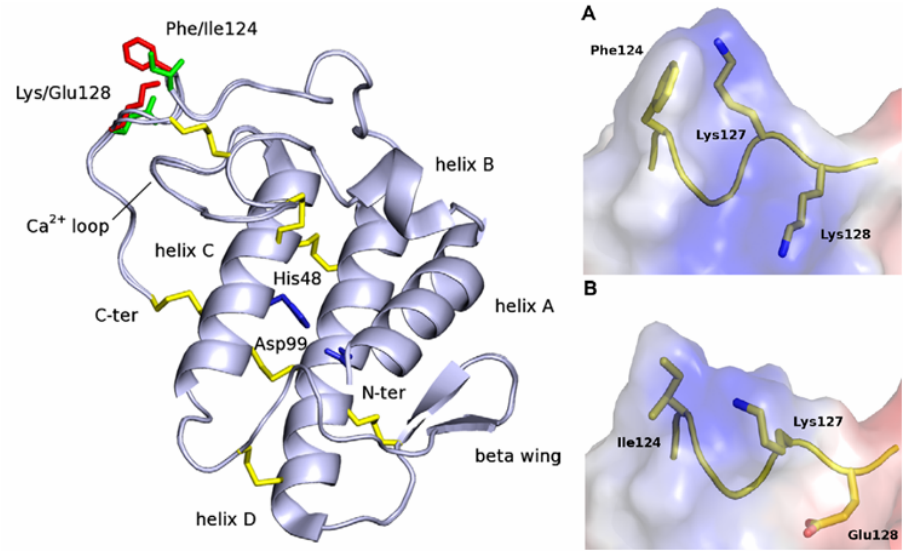

Figure 4. Superposition of tridimensional structures of AtxA and AtxC (left). AtxA and AtxC differ in their primary structures only on positions 124 and 128. The amino acid residues on these positions in AtxA are shown in red (Phe124 and Lys128) and in AtxC in green (Ile124 and Glu128). Figures A and B display structural detail in which both molecules differ from each other and the different charge distribution in this area (blue: positive, red: negative). Figures are reproduced from F.A. Saul et al., *Journal of structural biology*, 2009, doi:10.1016/j.jsb.2009. 10.010.

In 2009, we also continued our research on the role of $sPLA_2$s in the development and progression of breast cancer and the role of endogenous $sPLA_2$s in the (peripheral) nervous system, a specific target of the snake venom neurotoxic $sPLA_2$s.

Other pharmacologically active components from natural toxins

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 2009 we started the work on preparation of recombinant new type of conotoxin that possess more disulfides than all characterized conotoxins. We continued analysis of the venom proteome focusing on the components with molecular masses higher than 10 kDa and identified several new molecules. With structural analyses we participated at the description of the two novel conopeptides of the αD super-family. They selectively inhibit some subtypes of neuronal nicotinic acetylcholine receptor (S. Kauferstein et al., *Toxicon*, 54 (2009), 295–301). Based on the structure of RNA acquired from different *Conus* species we performed a phylogenomic analysis.

Together with the colleagues from the Biotechnical Faculty of the University of Ljubljana we characterized a new cytolysin from the venom of the sea anemone *Urticina crassicornis*. This toxin binds selectively to membranes enriched in cholesterol and sphingomyelin (A. Razpotnik et al*., Toxicon*, 53 (2009), 762–769).

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

Functional genomics is a field of molecular biology focused on describing function of genes in the genome and their interactions. One of the areas of functional genomics that develops the fastest is imaging-based phenotyping for the analysis of gene function. In collaboration with a research group from the University of Graz, Austria, we have developed a method and published a paper in which we describe a novel approach for linking high resolution imaging data with a genome-wide approach to understand the biogenesis of peroxisomes – organelles with an important role in the energy metabolism of cells – in the yeast *Saccharomyces cerevisiae* (H. Wolinski et al., *Journal of proteome research*, 8 (2009), 20–27) (Figure 5). In the future, such experimental approaches will enable much more detailed analyses of molecular mechanisms of action of drugs and different cellular perturbers of also mammalian cells.

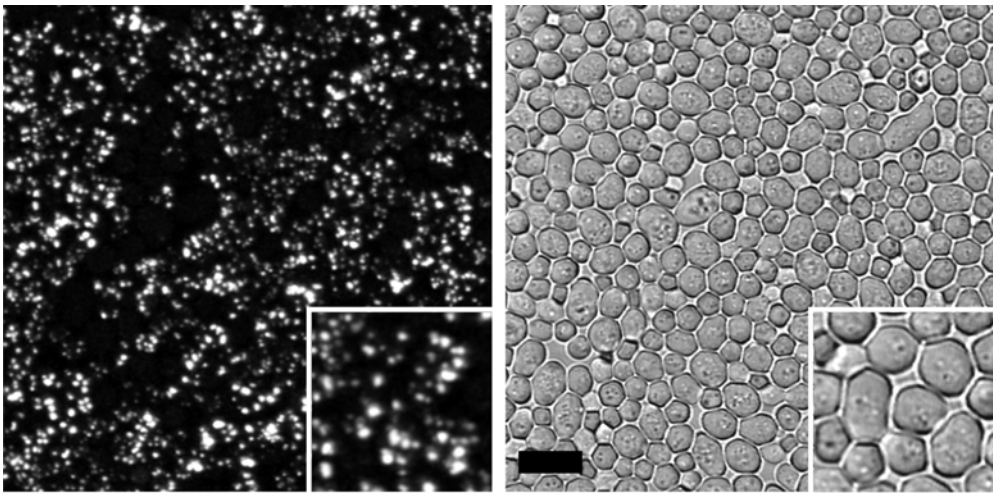

Figure 5. Yeast *S. cerevisiae* peroxisomes marked by a fluorescent marker (left) and corresponding cells imaged with transmission imaging (right). The image represents the phenotype of one of the 4740 strains that have been analyzed in the study; imaging of all strains required merely 3 days. The scale bar represents 10 μm. The figure is reproduced from H. Wolinski et al., *Journal of proteome research*, 8 (2009), 20–27.

Peroxisomes are organelles whose communication with other cell constituents is not well understood. Pex11 protein is involved in peroxisome proliferation both in yeast and mammalian cells, but its molecular mechanism of action is not known. Using DNA microarrays for transcriptome study, and high-throughput genetics and chemogenomics methods for genetic interactome study we proposed a model for the role of Pex11 in the regulation of energy metabolism, as a function of the availability of sugars or fats as the cellular energy source. This result has a potentially important influence in the development of new drugs and prophylaxis regimens for type 2 diabetes and other metabolic syndrome-related diseases.

In drug design and development, one of the main challenges is early identification of side-effects, which can significantly decrease the costs of research and development in the pharmaceutical industry. Development of chemogenomics methods enables the use of simple model organisms, such as yeast *S. cerevisiae*, as relevant *in vivo* systems for predicting molecular mechanism of action of drugs/chemicals globally. We have developed a new algorithm for combining chemogenomic and gene-interaction data that is based on local profile similarity and whose accuracy of target prediction for drugs or chemicals is superior to already available methods.

In collaboration with the research group from the Faculty of Computer and Information Sciences of the University of Ljubljana, we developed also an original computing method to study the structure of promoter regions in genes. We demonstrated the utility of the method in the analysis of gene expression data on budding yeast *S. cerevisiae* where cells were induced to proliferate peroxisomes (T. Curk et al., *Methods of information in medicine*, 48 (2009), 229–235).

Evolutionary genomics of transposable elements and functional studies of retrotranspo-sons

The cystatin superfamily comprises cysteine protease inhibitors that play key regulatory roles in protein degradation processes. Although they have been the subject of many studies, little is known about their genesis, evolution and functional diversification. A comprehensive survey of the cystatin superfamily, using the extensive genomic, proteomic and transcriptomic data for Archaea, Bacteria and Eukaryota, has provided new insights into their origin, evolution and classification. We have identified in silico the full complement of the cystatin superfamily in more than 2100 prokaryotic and eukaryotic genomes. The analysis of numerous eukaryotic genomes has provided strong evidence for the emergence of this superfamily in the ancestor of eukaryotes. Only two ancestral lineages, the stefins and the cystatins, exist in bacterial and eukaryotic genomes. In addition, 20 vertebrate-specific and three angiosperm-specific orthologous families have been discovered. In vertebrate orthologous families, the prevailing trends were loss of the ancestral inhibitory activity and acquisition of novel functions in innate immunity. Our analysis suggests that bacterial cystatins and stefins originated by horizontal transfer from the eukaryotic hosts, constituting a rare case of horizontal transfer from eukaryotes to bacteria. Bacterial cystatins and stefins may be emergency inhibitors that enable survival of bacteria in the host, defending them from the host's proteolytic activity. This study challenges the current view on the classification, origin and evolution of the cystatin superfamily and provides valuable insights into their functional diversification. The findings of this comprehensive study provide guides for future structural and evolutionary studies of the cystatin superfamily as well as of other protease inhibitors and proteases (D. Kordiš in V. Turk, *BMC evolutionary biology*, 9 (2009), 266).

The large amount of recently accumulated genome-wide data on transposable elements (TEs) in diverse lineages of sauropsids has provided a timely opportunity to review the current knowledge about transposable elements of sauropsids in their genomic context. TEs have profound effects on the structure, function and evolution of their host genomes. Our knowledge about these agents of genomic change in sauropsids, a sister group of mammals that includes

all extant reptiles and birds, is still very limited. Invaluable information concerning the diversity, activity and repetitive landscapes in sauropsids has recently emerged from analyses of the draft genomes of chicken *Gallus* and lizard *Anolis* 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 young, active TE families, the extant avian genomes have very few active TE lineages. Most reptilian genomes possess quite rich TE repertoires that differ considerably from those of birds and mammals, being more similar in diversity to that of lower vertebrates (D. Kordiš, *Cytogenetics genome research*, in press).

Human APOBEC3G (hA3G), a member of the AID/APOBEC family of deaminases, is a restriction factor for human immunodeficiency virus (HIV). In the absence of the viral Vif protein hA3G is packaged into virions and during reverse transcription in a recipient cell it deaminates cytosines, leading to G to A hypermutation and inactivation of the viral DNA. Unlike humans, who carry seven APOBEC3 genes, mice only carry one, mA3. Thus the role of mA3 in restriction of retroviral infection could be studied in mA3 -/- knockout mice, where the gene is inactivated. M-MuLV-infected mA3 -/- mice showed substantially higher levels of infection at very early times compared to wild-type mice (ca. 2 logs at 0-10 days), particularly in the bone marrow and spleen. Restriction of M-MuLV infection was studied *ex vivo* in primary bone marrow-derived dendritic cells (BMDCs) that express or lack mA3, using an M-MuLV-based retroviral vector expressing enhanced jellyfish green fluorescent protein (EGFP). The results indicated that mA3 within the virions as well as mA3 in the recipient cell contribute to resistance to infection in BMDCs. Finally, M-MuLV-infected mA3 +/+ mice developed leukemia more slowly compared to animals lacking one or both copies of mA3 although the resulting disease was similar (T-lymphoma). These studies indicate that mA3 restricts replication and pathogenesis of M-MuLV *in vivo* (A. Low et al., *Virology*, 385 (2009), 455–463).

Members of the apolipoprotein B mRNA editing complex polypeptide 1-like (APOBEC) family of enzymes exhibit inhibitory activity against a variety of exogenous and endogenous retroviruses including retrotransposons, such as long interspersed element 1 (LINE-1). Indeed, human APOBEC3A, APOBEC3B, and APOBEC3F inhibit retrotransposition of human LINE-1, mouse IAP and MusD retrotransposons. In our study, we examined whether the inhibitory effect of APOBEC3 proteins correlates with APOBEC3 ability to bind the LINE-1 ORF1 protein. We examined the interactions between the LINE-1 ORF1 protein and the most potent LINE-1 retrotransposon inhibitors, human APOBEC3A and APOBEC3B, by immunofluorescence and immunoprecipitation. Although human APOBEC3A shows the highest inhibitory potency against LINE-1 retrotransposon, no direct interactions were identified either by immunofluorescence or by co-immunoprecipitation. APOBEC3B binds to LINE-1 ORF1 protein, yet no co-localization was detected. We concluded that APOBEC3 proteins interfere indirectly with the LINE-1 retrotransposition pathway, probably through interference with RNA targeting (N. Lovšin and B.M. Peterlin, *Annals of the New York academy of sciences*, 1178 (2009), 268–275).

Other subjects

In 2009 we also collaborated at several projects out of the thematic scope of our department or the programme group "Toxins and biomembranes".

We performed structural analysis of trypsin-specific inhibitors isolated from the basidiomycete *Clitocybe nebularis* at the Department of Biotechnology at the Jožef Stefan Institute. They have regulatory and defensive functions in this fungus (P. Avanzo et al., *Microbiology* (2009), doi: 10.1099/mic.0.032805-0).

By analysis of the DNA and RNA samples of patients with unipolar depression we participated at the pharmacogenetic study of alternative antidepressant response of these people (R. Uher et al., *American journal of psychiatry,* in press).

In the quantification study of coenzyme Q10 and cholesterol in fractionated chicken-breast tissue we were preparing a homogenous mitochondrial fraction from the cells (P. Jazbec et al., *Journal of planar chromatography*, 22 (2009), 395–398).