Final Report - CONBIOS (330486)

The objective of this grant was to elucidate the molecular mechanisms of oxidative folding and modification of conopeptides, small-disulfide-rich peptides expressed in the venom of marine cone snails. Achieving this required a large-scale sequencing effort of venom gland transcriptomes of a diverse set of cone snail species belonging to a variety of lineages and developing bioinformatics tools to efficiently mine these datasets. By doing so this project not only discovered a novel class of enzymes critical for conopeptide folding (conotoxin-specific protein disulfide isomerase (csPDIs)), but also identified thousands of new conopeptide sequences with biomedical potential, including a novel family of insulins that is currently being investigated as a drug lead for the treatment of diabetes.

Venom peptides from marine cone snails (conopeptides) have received much attention over the last few decades due to their biological activity and extraordinary diversity. Each of the ~700 species of cone snail expresses a characteristic repertoire of 100-400 peptides leading to an estimated 200,000 different bioactive compounds in this animal genus. Most conopeptides target receptors, ion channels and transporters of the central and peripheral nervous system. Receptor subtype specificity has been demonstrated for almost all known conopeptide resulting in their wide use in neuroscience research, pharmacology and drug development. An increasing number of conopeptides also show therapeutic potential, particularly as analgesics. For example, the omega-conopeptide MVIIA (from *Conus magus*) is an approved drug for the treatment of pain.

Pharmacological testing and molecular characterization of novel conopeptides requires large amounts of peptides, which are commonly generated by chemical synthesis. However, chemical synthesis often results in very low functional yields and aggregated products, mostly due to the presence of multiple cysteine residues, which must oxidize to form the correctly folded disulfide isomer. Conopeptides that are difficult to make are often found in high abundance in cone snail venoms suggesting that the venom gland has evolved specialized “helper” proteins for the proper folding of these diverse peptides. Thus, understanding the molecular mechanisms of conopeptide folding in the venom gland is likely to improve current chemical synthesis approaches and could inform on the generation of an expression system for the large-scale production of conopeptides for pharmacological studies.

Our initial observations on the venom glands of two cone snails, *Conus geographus* and *Conus bullatus*, suggested the presence of a previously unknown gene family of protein disulfide isomerase (PDI), an enzyme that is of fundamental importance for protein folding in the endoplasmic reticulum. Following this discovery the venom gland transcriptomes of 16 cone snail species were sequenced using next-generation Illumina technologies and mined for PDI expression. This large effort confirmed that cone snails evolved a novel class of PDIs that we refer to as conotoxin-specific PDI (csPDI). This gene family undergoes accelerated evolution with high mutation rates in regions known to be important for catalytic activity. Functional characterization of several recombinant csPDIs and canonical PDI from *C. geographus* demonstrated that members of the csPDI family significantly and differentially accelerated the folding rates of various conopeptide substrates. These findings were presented at several international meetings and published in PNAS in March 2016 (Safavi-Hemami et al.). Following this discovery we were able to generate a recombinant expression system where newly identified conopeptide sequences are co-expressed with csPDI in a bacterial strain optimized for disulfide-rich peptide expression. Our preliminary data suggests that this system can efficiently produce a large number of novel conopeptide sequences at sufficient amounts for pharmacological and structural evaluation.

Simultaneously our investigations into the proteins expressed in the venom gland of *C. geographus* led to the identification of a highly abundant venom insulin that shared high sequence similarity with fish insulin, the prey of this cone snail species. This was an unexpected and exciting finding, as insulin had never been reported in any animal venom before. We hypothesized that this specialized insulin is utilized to facilitate prey capture through inducing a hypoglycemic shock in the prey. Experiments investigating this hypothesis were conducted and confirmed that venom insulins evolved to facilitate prey capture. These findings were published in *PNAS* in 2015 (Safavi-Hemami et al.) and received international media attention, including news features in *National Geographic*, *the Guardian*, *the Examiner* and *die Welt*. Analysis of the transcriptomes of nearly 20 additional cone snail species revealed that venom insulins are widely distributed in the venom of cone snails but that only certain groups of fish-hunting species utilizes insulins that are similar to fish and human insulins. Our findings on the evolution of venom insulins were published in *Molecular Biology and Evolution* in 2016 (Safavi-Hemami et al.).

Following these discoveries we noticed unique structural features of the fish-like venom insulin, including Con-Ins G1 from *C. geographus*, that could potentially render it an ideal candidate for the development of a novel class of fast-acting insulin analogs for the treatment of diabetes. In order to further investigate this we determined the high-resolution X-ray structure of Con-Ins G1 and showed that it was highly active at the human insulin receptor. These findings were published in *Nature Structural and Molecular Biology* in 2016 (cover image; Menting et al.) and initiated a series of follow-up studies that are likely to result in additional discoveries in this area of research in the near future.