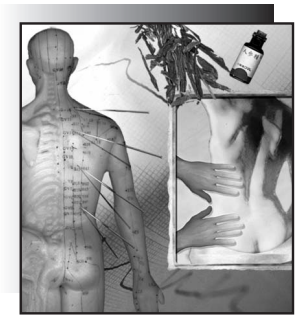

Molecular Prospecting for Drugs from the Sea

Isolating Therapeutic Peptides and Proteins from Cone Snail Venom

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Needing to disable prey or predators quickly, most animals use venom for capture or protection. Venom has therefore evolved to suit these functions; it consists of agents that interfere with neuronal and muscular circuitry. But how does this benefit us? Clearly, some of these agents act in a way that can be therapeutic. Therefore, these venom components are potentially valuable because they could be a rich source of therapeutic agents.

Our studies seek new drugs from the marine environment, with a particular focus on venomous animals. One animal of particular interest is the marine cone snail, *Conus*. This is a genus of predatory animal with a highly evolved venom and delivery apparatus. These remarkable creatures provide an example of the rich diversity of therapeutic molecules still to be discovered in marine species. In this article, we describe why these are valuable animals for medical research and the methods we have used to realize the therapeutic potential of their venom.

Conus is a highly successful genus of predatory marine snail. There are approximately 500 species in this genus [1]. This makes *Conus* one of the most successful living marine animals [2]. The success of *Conus* has been attributed to the evolution of a potent venom [5] and a hollow harpoon-like radular tooth to deliver it [6]. The venom is a complex mixture of 50–200 distinct, biologically active components (Figure 1) that act to immobilize prey.

Specimens of the genus *Conus* have caused injuries, and even death, to man. In fact it was when describing an example of a human injury from the sting of *C. aulicus* in 1848 that Adams described the complex venom and highly sophisticated delivery apparatus for the first time [7]. Such human injuries have predominantly been attributed to piscivorous species of *Conus* [8]. One species in particular, *Conus geographus* has caused a number of human fatalities, including one on the Great Barrier Reef.

Different species of modern *Conus* occupy a variety of habitats—including rock, coral, and sand—and can be found anywhere from intertidal pools to deep water. They predominantly inhabit tropical waters [1]. They are nocturnal predators living on marine worms, hemichordata, cephlapods, gastropoda, bivalves, crustacea, and fish [3], [4].

Conus Venom

Components of the venom include both proteins and peptides. Of these, peptides are the most studied and perhaps the most interesting. Due to their small size and stability, peptides are suitable for a number of medical and pharmacological applications. The peptides from *Conus* venom (conopeptides) have been classified according to their secondary structure into two main groups. The larger of these groups contains disulfide-rich conotoxins, characterized by two or more disulfide bonds that serve to stabilize the conotoxin structure. The second group is characterized by either one single disulfide bond or a total lack of disulfide bonds. Our studies have concentrated on the former of these groups, the conotoxins.

As shown in Figure 2, some of the peptides found in *Conus* venoms are known to be antagonists at nicotinic acetylcholine receptors (α-, αA-, and ψ-conotoxins), others antagonists at voltage-gated sodium-ion channels (μ-, μO-conotoxins), agonists that inhibit inactivation at voltage-sensitive sodium channels (δ-conotoxins), antagonists at voltage-sensitive calcium channels (ω-conotoxins), antagonists at potassium channels (κ- and κA-conotoxins), inhibitors of 5 hydroxytryptamine (HT) receptors (σ-conotoxins), inhibitors of noradrenaline uptake (χ-conotoxins), antagonists at N-methyl-D-asparate (NMDA) receptors (conantokins and conodynes), inhibitors of α1-adrenoceptors (ρ-conotoxins), or inhibitors of neurotensin receptors (conorfamides and contulakins). Every species of *Conus* has the following at its disposal to capture prey: the venom apparatus, a cocktail of biologically active toxins in its venom, and the 50 million years of evolution that lead to the development of its arsenal. But why should we be so interested in these conotoxins?

There are a great number of toxins in *Conus* venom; at least 25,000 components make up the entire library of *Conus* toxins, and, of these, only a few hundred have been identified. Also, these conotoxins display a remarkable specificity for their target receptors. Finally, the conotoxins are relatively small in size and inherently stable in vivo. The real value of *Conus* venom is a combination of all these factors. We are trying to harness the acute specificity generated through over 50 million years of evolution, in combination with the wealth of individual venom components from *Conus*, to develop new tools and therapies for medical research.

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A number of conotoxin families have been proposed as possible therapeutics for a range of disease states; ρ-conotoxins, active at antagonising α_1 adrenergic activity, have been reported to have potential for the treatment of urinary and cardiovascular conditions, mood disorders, and pain and inflammation [9]. Another family of conotoxins with therapeutic potential is the τ-conotoxins, part of the T-superfamily of conotoxins. While no definitive target for these conotoxins has been identified, it has been proposed that they may target presynaptic Ca^{2+} channels or act on G protein-coupled presynaptic receptors via another mechanism. Some of these peptides may have therapeutic applications in managing pain [10].

The κ-A conotoxins are a small family of conotoxins targeting voltage-gated K^+ channels. Based on the ability of the peptide to inhibit the flow of K^+ through voltage-gated K^+ channels, the κ-A conotoxins may have applications in the treatment of demyelinating diseases such as multiple sclerosis.

The ψ-conotoxins, bind to the outer vestibule of the pore formed by muscle-type nicotinic acetylcholine receptors, functionally blocking the channel. These peptides have potential as therapies that block skeletal muscle function, as with the treatment of cerebral palsy.

The χ-conotoxins are the latest addition to the list of conotoxins with therapeutic potential. This class of conotoxins was shown to inhibit noradrenaline transport in rat vas deferens [11], and the reported utility of these peptides is in the treatment and prevention of pain [11], [12].

The next two families of conotoxins both target voltage-gated sodium channels. It is claimed that μ-O conotoxins have utility as local anaesthetics. Alternately, δ-conotoxins have been shown to slow down the inactivation of sodium channels. They may have utility in improving impulse conductance and the transmission of action potentials through demyelinated nerves in multiple sclerosis patients and patients with spinal cord injury.

The κ-conotoxins irreversibly block K^+ channels and could be important in the treatment of Alzheimer's disease and myasthenia gravis. Bromosleeper conotoxins are antagonists of the 5-HT_3 ligand-gated ion channels [13]. These conotoxins are potentially useful as sleep-inducing agents and as adjuncts to anesthesia. The μ-conotoxins potently block sodium channels and have been implicated as potential therapeutics for addressing pain states.

One of the most interesting classes of conotoxins with therapeutic potential is the class containing the so-called ω-conotoxins that block Ca^{2+} channels. The N-type Ca^{2+} channels in particular are of crucial importance in the neural pathways responsible for the transmission of pain. As such, these conotoxins are useful for producing analgesia [19]. Two conotoxins in this family are of particular interest. The ω-conotoxin MVIIA, was shown to effectively produce antinociception in rats in a number of models of pain states [14]–[17]. The conotoxin MVIIA, now known as ziconotide, has undergone clinical trials, displaying a significant reduction in pain in a number of different patient populations [18].

The ω-conotoxin CVID has also been investigated as an analgesic. The ω-conotoxin CVID also targets Ca^{2+} channels. This peptide displays even greater selectivity for N-type Ca^{2+} channels over P- and Q-subtypes in binding assays [19]. This increased specificity for the N-type Ca^{2+} channels is believed to be important for reducing the negative side effect that ziconotide displays.

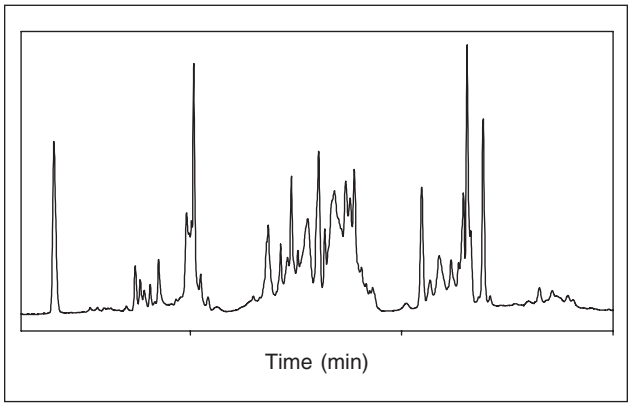

Fig. 1. Reversed-phase high-performance liquid chromatography of venom from *Conus textile*. Peaks are detected at 215 nM.

Super Family	Cysteine Arrangement	Class	Molecular Target	Example*
A	CC-C-C CC-C-C-C-C	α κA	nAChR Antagonist K^+ Channel Antagonist	α-EpI κA-SVIA
O	C-C-CC-C-C	δ	Na^+ Channel Agonist	δ-TxVIA
	C-C-CC-C-C	ω	Ca^{2+} Channel Antagonist	ω-MVIIA
	C-C-CC-C-C	μO	Na^+ Channel Antagonist	μO-MrVIA
	C-C-CC-C-C	κ	K^+ Channel Antagonist	κ-PVIIA
M	CC-C-C-CC	μ	Na^+ Channel Antagonist	μ-GIIIA
	CC-C-C-CC	ψ	nAChR Antagonist	ψ-PIIIE
T	CC-CC	ε	Ca^{2+} Channels	ε-TxIX

Fig. 2. Molecular targets for conotoxins: Ep1 (23); SVIA (38); TVIA (36); MVIIA (39); MrVIA (40); PVIIA (37); GIIIA (41); PIIIE (42); and TxIX (43). When injected by the harpoon-like radular tooth, venom blocks various ion-channels and receptors in the neuromuscular system (31).

Mining *Conus* for New Therapeutic Agents

The source of venom diversity in cone shells is genetic. Genes determine the primary amino acid sequence of each peptide. For conotoxins, the primary sequence varies significantly both between species and between the different families of conotoxins. In general, the cystinyl residues involved in disulphide bridges are conserved for each conotoxin class (Figure 3); the cystinyl residues are essential for biological functions of these peptides. The other residues are hypervariable among conotoxins from different species. The variation in sequence produces subtle differences in biological activity. Such genetic diversity leads to

something in excess of 25,000 different compounds with therapeutic potential in *Conus* venoms.

So how do we efficiently interrogate this diverse resource? Because the peptides are produced in the venom apparatus, the venom apparatus is the primary source of these peptides in nature. However, cone shells are not easy to find. Cone shells inhabit many of the world's most precious marine ecosystems. They are nocturnal and seasonal in distribution and live buried in sand and under rocks. They are difficult, if not impossible, to breed in captivity and are generally difficult to milk for venom. Even with successful captive breeding, specimens do not yield the same venom with repeated milking or sustained periods in captivity.

Because access to the venom duct requires destruction of the specimen, this will have an undesirable impact on the ecosystem. In addition, venom peptides obtained from the duct commonly contain an array of chemical modifications. For the snail, the modifications make small alterations to the structure and activity of the toxins. For us, the modifications can make chemical synthesis much more difficult. A number of such modifications have been identified in *Conus* peptides.

With some 10% of *Conus* peptides estimated to contain γ-carboxyglutamate, this modification is emerging as one of the more common posttranslational modifications occurring in *Conus* peptides; γ-carboxyglutamate facilitates the formation of an α-helix in conopeptides [20].

An unusual modification of *Conus* venom peptides is the conversion of an L- to a D-amino acid. An example of this is the family of contryphans. In most of these peptides, one of two tryptophan residues are epimerised from an L-tryptophan to a D-tryptophan. The biological significance of this modification is unknown. Similar conversions have been described in the opiate analogues, the deltorphins from frog skin, and the spider toxins ω-agatoxin (see the review by Kreil [21]).

The hydroxylation of proline residues is also common for conotoxins. The role of this modification, in the interaction of a conotoxin with its cognate receptor, is unclear. In other systems, like triple-helical collagen I, the role of proline hydroxylation is well-defined in collagen stabilization. Collagen molecules with unhydroxylated proline residues display increased flexibility and reduced melting temperatures [22]. This has obvious implications for the shape and stability of a small molecule like a conotoxin, and may influence the binding of a peptide with its cognate receptor.

Sulfotyrosine has been identified in the α-conotoxins EpI from *C. episcopatus* [23], and PnIB from *C. pennaceus* [24]. Tyrosine sulfation was not observed to confer any functional benefit upon binding of the α-conotoxin EpI to bovine nicotinic acetylcholine receptors [23]. The role of sulfation has not been investigated further in the context of conotoxin-receptor recognition.

Protein tyrosine sulfation is emerging as a common post-translational modification in eukaryotes. Many of these tyrosine-sulfated proteins are involved in protein-protein interactions that may be influenced, at least in part, by the sulfate group [25]. Sulfation is required for agonist recognition by glycoprotein hormone receptors, and is a key modulator of protein-protein interactions that mediate fractalkine-induced cell adhesion [26]. While sulfation of the tyrosine residue on EpI did not seem to be functionally important to the activity of the α-conotoxin at bovine nicotonic receptors, sulfation clearly plays a major role in many receptor-ligand binding interactions.

α-Conotoxins

(Linked by Two Disulfide Bonds)

3:5 Loop—Block Muscle-Type nAChRs

ECC-NPACGRHYS--C	GI	Conus Geographus
ICC-NPACGPKYS--C	SI	Conus Striatus

4:7 Loop—Block Neuronal-Type nAChRs

GCCSDPRCAWR - - - - C	ImI	Conus Imperialis
GCCSDPRCNMNNPDYC	EpI	Conus Episcopatus
GCCSLPPCAANNPDYC	PnIA	Conus Pennaceus
GCCSLPPCALSNPDYC	PnIB	Conus Pennaceus

Fig. 3. Amino acid sequences of conotoxins.

A common modification to occur to conopeptides is the bromination of tryptophan to 6-L-bromotryptophan. This modification has been identified in bromocontryphan, the bromosleeper peptide, the bromoheptapeptide, and σ-conotoxin GVIIIA [27], [28], [13]. No function has yet been attributed to this modification in any of the four peptides.

Glycosylation is another posttranslational modification observed in *Conus* venom with two different families of O-linked glycopeptides that have been identified to date. The κ-A conotoxin SIVA contains a pentasaccharide O-linked to a serine residue, while contulakin G contains a disaccharide. The efficacy of both peptides is significantly greater in the glycosylated form than in nonglycosylated analogues [29]. Such posttranslational modifications have been seen as a major stumbling block to the chemical production of these modified peptides and of their folding into active conformations. To synthesize peptides with the various modifications is both a challenge and an expense and can even be an impediment to the achievement of a biologically active conformation in vitro.

In summary, venom-duct peptides in their raw or native state are

- ➤ structurally diverse
- ➤ hard to synthesize chemically, especially with the above modifications
- ➤ hard to obtain without killing the cone snail.

To minimize the environmental impact, we have sought to use genes instead of native peptide isolation. In doing so we have minimized the number of *Conus* specimens required. One or two specimens is enough to create and store large libraries of conotoxin genes.

In the process, we have demonstrated [32] that the deduced primary amino acid sequence can provide sufficient information for obtaining a biologically active peptide. The conotoxins we identified were easy to synthesize and did not need the chemical modifications for activity. In summary, we have chosen to isolate genes as an alternative route to amplify both the effectiveness of the bio-prospecting and also the cost-efficiency of the downstream synthesis.

Finding the Relevant Genes

Fortunately the genes for conotoxins are orderly and readily adapted to mining. While there is hypervariability in the conotoxin sequence, each conotoxin peptide is produced as a precursor protein that has highly conserved regions.

To isolate the conotoxin precursors, we need to know something about the conotoxin genes. The analysis of cDNA sequences has demonstrated that conotoxins are synthesized as precursors. These are approximately three times larger than the secreted, mature peptide [36], [30], [31]. It is now known that all conotoxins are synthesized as larger precursors in the epithelial cells of the venom duct and are presumably processed in the endoplasmic reticulum to produce the mature peptide that is secreted into the venom duct. The precursor contains three main regions, the preregion (or, signal sequence), the proregion, and the mature region that will form the conotoxin when fully processed and cleaved from the precursor molecule (Figure 4).

The signal sequence is believed to direct translocation of the propeptide to the endoplasmic reticulum where it is cleaved by signal peptidase [33]. It has been proposed that the remaining propeptide sequence may help direct the disulfide-coupled folding of the conotoxins in the lumen of the endoplasmic reticulum, prior to being removed to release the functional toxin [31]. It has also been proposed that the proregion has a role in protein folding. For the ω-conotoxins MVIIA and GVIA, however, the experimental evidence refutes this, both thermodynamically and kinetically [33]. It is also unlikely that the proregion promotes interaction between the enzyme protein disulfide isomerase and the conotoxin [33]. Other proposed functions for the pre-proregion, such as enhancing the solubility of the nascent chain, promoting interactions with other cellular factors, directing posttranslational modifications, or directing the propeptide to secretory granules prior to entering the venom [33], are still to be supported by experimental evidence.

We conclude from our work that a significant proportion of conotoxin peptides, when synthesized in vitro, are capable of both folding and forming intramolecular disulphide bridges—even in the absence of pre-proregions—producing a biologically active conformation.

In the modern era of molecular biology, genes with common conserved sequences at each end are particularly suited to discovery by polymerase chain reaction (PCR), a popular technique for the identification of rare gene products [34]. A minimal quantity of starting material is required, and when combined with its intrinsic sensitivity, PCR is a powerful tool to identify poorly expressed messengers.

Messenger RNA (mRNA) is a particularly suitable starting material for gene identification by PCR. Compared to genomic DNA, mRNA contains only the coding exonic sequence and not the large introns that have been reported for conotoxin genes [35]. Another advantage of using mRNA in preference to genomic RNA is that only a proportion of the genome will be expressed in any one cell. By selecting the right tissue as a starting material, it is possible to create a sample of cDNA, enriched for a particular transcript. Cells actively producing conotoxins, like the cells of the venom duct, contain large amounts of conotoxin-encoding mRNA. The limitation of this method is that only those genes expressed at the time of processing can be amplified.

One example of PCR is: α-conotoxin genes encode a very distinctive preregion characterized by a methionine-rich amino-terminal end. This conserved region provides the sequence from which to design an α-conotoxin specific primer. Similarly, transcripts from the O-superfamily share a conserved (and quite distinct) preregion at the amino-terminal end of the precursor. A specific primer was designed to hybridize to this region of the conotoxin precursor mRNA to amplify the encoding DNA in a PCR reaction. By obtaining the sequences of the cDNAs this way, the sequences of the encoded conotoxin peptides are predicted. Many novel conotoxin sequences have been identified in our laboratory using this method.

A key feature of the DNA-based approach is that conotoxins of a particular class, with very different physical properties, can be identified from PCR reactions, primed by sets of relatively universal oligonucleotides. The sensitivity of PCR also means that sequences can be identified from small amounts of starting material. This provides a twofold benefit: 1) only small numbers of cone snails need to be sacrificed to provide enough starting material for cDNA synthesis, and 2) less abundant peptides can also be identified. DNA-based techniques may prove vital in identifying sequences that exhibit interesting biological activity but would otherwise remain undetected.

The final step in identification of the lead compounds in our case has been to screen predicted peptide

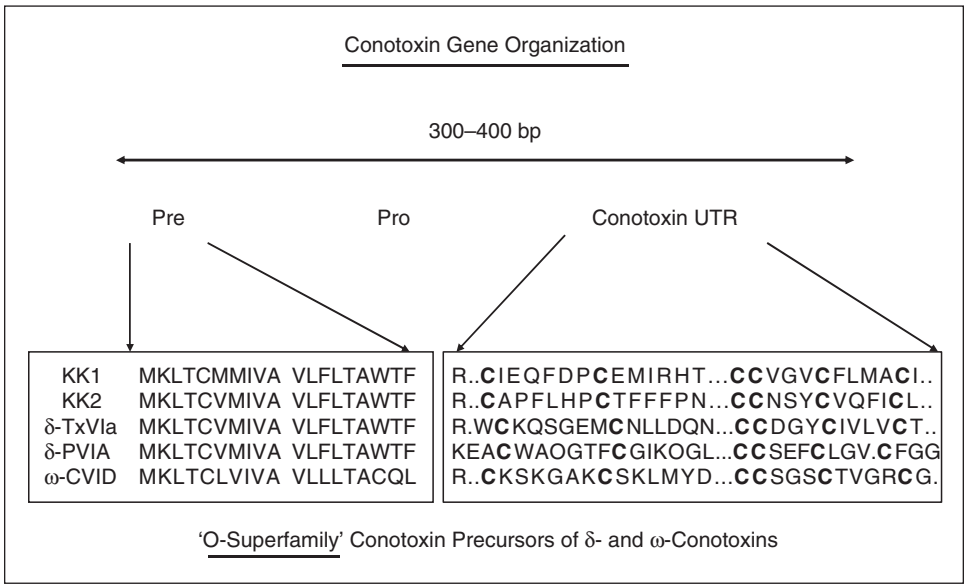

Fig. 4. The precursor structure of conotoxins: The conserved preregion and the functional conotoxin region are joined at the time of synthesis by a partially conserved proregion (not shown).

sequences in relevant bioassays. This is a three-step process in which the predicted peptide is first synthesized by standard peptide chemistry, then oxidized to form the key disulphide bridges, and finally tested in a relevant screening bioassay. This is in fact the strength of the DNA approach—simple peptide synthesis combined with a sensitive bioassay as the primary screen. This approach detects those peptides that spontaneously form a functional three dimensional (3-D) structure under the conditions of primary amino acid chain synthesis and oxidation.

An example of the success of this DNA approach is the peptide with potential analgesic properties recently discovered in cone venom [32]. Peptide Vc1.1, a disulphide-bonded peptide of 16 amino acids in length whose structure was predicted solely from its DNA sequence. Following simple chemical synthesis and oxidation, it spontaneously formed an active antagonist of the neuronal nicotinic acetylcholine receptor. In subsequent bioassays, this novel and stable conopeptide has proven to suppress pain responses in vivo [32].

Conclusions

DNA- or gene-based mining can, when coupled with sensitive bioassays, be an environmentally friendly method for discovering novel therapeutic peptides from marine sources. Simple peptide synthesis combined with a sensitive bioassay is the primary screen. This detects those peptides that form a functional 3-D structure spontaneously under the conditions of primary amino acid chain synthesis and oxidation.

The screening process therefore selects lead compounds that are simple to manufacture. Spontaneity of folding may also indicate greater stability of the structure under physiological conditions, and the selection procedure, therefore, may pick those peptides with greater inherent stability in vivo, a further bonus that may be related to the success of delivery and survival of the lead compounds when used therapeutically.

Ken Gayler is an associate professor and the head of biochemistry and molecular biology at The University of Melbourne, Australia, with research laboratories located in the Bio21 Research Institute. His interest in molecular techniques such as PCR to characterize poorly expressed gene products is being used to advantage to identify novel conopeptides from Australian cone shells.

David Sandall is a biochemistry postgraduate student currently completing his Ph.D. on the characterization of novel conopeptides from Australian cone shells using molecular and proteomic techniques. His research has contributed to the isolation of a number of novel sequences from the temperate cone, *Conus anemone*, and from tropical cones such as *Conus virgo*, *Conus victoriae*, *Conus textile*, and *Conus virgo*, among others.

David Greening, a Science Honours undergraduate in the Ken Gayler/Bruce Livett laboratories, is pursuing a Ph.D. in proteomics. During his Honours year, he fractionated neuropeptides from the venom of *Conus marmoreus* (collected from the Great Barrier Reef) and identified several novel peptides using mass spectrometry. Some were shown to have activity at the nicotinic acetylcholine receptor.

David Keays is a science and law graduate from Melbourne University, Australia, who is currently completing his D.Phil. in the field of neuroscience research in Oxford, England. During his Science Honours year in the Ken Gayler/Bruce Livett laboratories, he took on a project to study the conopeptides present in the venom duct of the Australian cone shell, *Conus victoriae*. This project led to the identification of conotoxin Vc1.1 which is currently being developed commercially by Metabolic Pharmaceuticals, Melbourne, Australia, as an analgesic for chronic neuropathic pain.

Megan Polidano recently graduated with a Ph.D from Bruce Livett's laboratory where she used molecular probes to characterize the tissue distribution of neuronal nicotinic receptor subunits and used alpha-conotoxins in competitio with the toxin probe, epibatidine, to characterize conotoxin binding sites in mammalian tissues. After a brief postdoctoral period at Cognetix in Salt Lake City, Utah, she returned to Australia and is continuing her research at the Pharmacy College, Melbourne.

Bruce Livett is an associate professor and reader in biochemistry and molecular biology at The University of Melbourne, Australia, and a malacologist, with research laboratories located in the Bio21 Research Institute. His interest in the mechanism of action of peptides as modulators of the neuronal-type nicotinic acetylcholine receptor led him to examine Australian cone shells as a source of novel compounds. He also maintains the Cone Shell and Conotoxin HomePage, an authorative and up-to-date resource at http://grimwade.biochem.unimelb.edu.au/cone/.

John Down is an organic chemist (retired) and malacologist, with a particular interest in peptide sequences and alignments. He contributed to the selection of likely candidates for solid-phase synthesis from among the large number of conotoxin sequences isolated by the molecular approach.

Narmatha Satkunanathan is currently completing her Ph.D. with Zeinab Khalil at the National Ageing Research Institute (NARI), University of Melbourne, Australia. Her research making use of animal models of human neuropathic pain has contributed significantly to our understanding of the analgesic properties of the conotoxins and their potential as therapeutics in the management of neuropathic pain conditions.

Zeinab Khalil is an associate professor and head of the biology research laboratories at the National Ageing Research Institute, associated with The University of Melbourne. She

has an M.D. and Ph.D. and is most interested in translating the results of bench science to clinical practice. She has a long-standing interest in pain mechanisms and in early detection of Alzheimer's. Her physiological expertise has been directed recently using a number of animal models of human neuropathic pain, to assess the potency of cone shell peptides as novel analgesics.

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