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Therapeutic applications of conotoxins that target the neuronal nicotinic acetylcholine receptor

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Abstract

Pain therapeutics discovered by molecular mining of the expressed genome of Australian predatory cone snails are providing lead compounds for the treatment of neurological diseases such as multiple sclerosis, shingles, diabetic neuropathy and other painful neurological conditions. The high specificity exhibited by these novel compounds for neuronal receptors and ion channels in the brain and nervous system indicates the high degree of selectivity that this class of neuropeptides can be expected to show when used therapeutically in humans. A lead compound, ACV1 (conotoxin Vc1.1 from *Conus victoriae*), has entered Phase II clinical trials and is being developed for the treatment for neuropathic pain. ACV1 will be targeted initially for the treatment of sciatica, shingles and diabetic neuropathy. The compound is a 16 amino acid peptide [Sandall et al., 2003. A novel α-conotoxin identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves in vivo. Biochemistry 42, 6904–6911], an antagonist of neuronal nicotinic acetylcholine receptors. It has potent analgesic activity following subcutaneous or intramuscular administration in several preclinical animal models of human neuropathic pain [Satkunanathan et al., 2005. Alpha conotoxin Vc1.1 alleviates neuropathic pain and accelerates functional recovery of injured neurons. Brain. Res. 1059, 149–158]. ACV1 may act as an analgesic by decreasing ectopic excitation in sensory nerves. In addition ACV1 appears to accelerate the recovery of injured nerves and tissues.

Keywords: Nicotinic receptor; Conotoxin; Pain

Two roads diverged in a wood, and I-

I took the one less travelled by,

And that has made all the difference.

Robert Frost

1. Introduction

Pioneering studies in the 1960s and 1970s by Bob Endean and colleagues paved the way for the discovery of novel pharmacologically active compounds from Australian marine molluscs. Endean studied the pharmacology of venoms from fish-hunting cone snails (Endean and Rudkin, 1963; Endean et al., 1979; Hawgood, 2005). Since then a

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number of Australian scientists have investigated cone snails as a source of novel therapeutic compounds to treat neurological disorders (Alewood et al., 2003; Livett et al., 2004; Gayler et al., 2005; Norton and Olivera, 2005). The α-conotoxins, a class of peptides that antagonise the neuronal nicotinic acetylcholine receptors hold such promise.

Peptide neurotoxins have historically been extremely useful for characterizing the nicotinic acetylcholine receptor (Chiappinelli, 1993; Rajendra et al., 2004) (Table 1). The best known is α-bungarotoxin from the Taiwanese-banded krait *Bungaris multicinctus* which was used by the early biochemists in affinity chromatography to isolate nicotinic acetylcholine receptors (nAChRs) associated with skeletal neuromuscular junctions—the so-called *muscle-type* nAChRs (Tsetlin and Hucho, 2003). The first α-conotoxins discovered, including α-GI from *Conus geographus* (Cruz et al., 1978) and SI from *Conus striatus* (Ramilo et al 1992; Zafaralla et al., 1988) have likewise served as useful tools to explore the pharmacology of *muscle-type* nicotinic acetylcholine receptors (Groebe et al., 1997; Hogg and Bertrand, 2004; Velez-Carrasco et al., 2004). *Muscle-type* nAChRs are pentameric ligand-gated ion channels with a subunit composition $(\alpha)_2\beta\gamma\delta$, whereas *neuronal-type* nAChRs are pentameric oligomers composed exclusively of α and β subunits, e.g. heterooligomeric α2β2, α3β4 and α2β4 and homooligomeric α7 and α9 (Nicke et al., 2004). There are, however, limited compounds that specifically target *neuronal-type* nAChRs.

A need arises for additional probes that target the *neuronal-type* nicotinic acetylcholine receptor so that their biochemical and pharmacological properties can be fully explored and to enable more

Table 1
Nicotinic acetylcholine receptor (nAChR) toxins from natural sources

Source	Genus/families	Chemical nature	Specificity	Reference
Agonists				
Plant	Tobacco	Alkaloid nicotine	Muscle and neuronal nAChRs	Yildiz (2004)
Blue-green algae	cyanobacterium *Anabaena flos-aquae*	Alkaloid (+)-Anatoxin-A	Muscle and neuronal nAChRs	Molloy et al. (1995)
Frog skin	Ecuadorian tree frog, Epibatides	Alkaloid Epibatidine	Muscle and neuronal nAChRs	Daly et al. (2000), Spang et al. (2000)
Antagonists				
Plant	*Chondodendron tomentosum*	Curare (d-Tubocurarine)	Muscle and neuronal nAChRs	Langley (1907), Chavez-Noriega et al. (1997)
Plant	*Erythrina*	Alkaloid (Erythroidine)	Muscle and neuronal nAChRs	Chavez-Noriega et al. (1997)
Plant	*Delphinium brownii*	Diterpenoid Methyllycaconitine	Muscle and neuronal nAChRs	Ward et al. (1990), Mogg et al. (2002)
Soft coral	*Lophogorgia pseudopterogorgia*	Diterpenoid Lophotoxins, pukalides and bipinnatins	Muscle and neuronal nAChRs	Abramson et al. (1988, 1989), Livett et al. (1991), Tornoe et al. (1995)
Marine sponges	*Luffariella geometrica and Spongi* sp.	sesterterpenes luffarins and cometins	Muscle and neuronal nAChRs	Dean et al. (1994), Down et al. (1995), Broxton et al. (1996)
Japanese Ivory Mollusc (mid-gut gland)	*Babylonia japonica*	Polyol (neosurugatoxin)	Muscle and neuronal nAChRs	Hayashi et al. (1984), Yamada et al. (1988), Bourke et al. (1988), Hong et al. (1992), Tornoe et al. (1995)
Snake venom	Elapidae and Hydrophidae	Polypeptides (a-neurotoxins. E.g. α-bungarotoxin) (Also κ-neurotoxins)	Muscle nAChRs predominantly	Chiappinelli (1993)
Cone snail venom	*Conus* (Piscivorous Molluscivorous and Vermivorous)	Small peptides (10–40 amino acids)	High specificity for muscle or neuronal nAChRs	Olivera and Cruz (2001), Terlau and Olivera (2004)

selective ligands to be developed. The development of selective compounds that target the neuronal nicotinic receptor could also provide novel therapeutic agents for the treatment of a range of neurological disorders that involve these receptors (Decker et al., 2004; Flores, 2000). These include neurological disorders such as epilepsy, Parkinson's and Alzheimer's disease, nicotine addiction and a range of neuropathic pain syndromes, for which present medications are inadequate and can have undesirable side effects (Dickinson, 2003). Whereas others had found that nicotinic receptor agonists such as nicotine and epibatidine were analgesic (Daly et al., 2000; Decker et al., 2004), perhaps through the release of endogenous analgesics such as the nociceptins in the CNS, we were led to consider that these strong agonists might induce nicotinic receptor desensitization and therefore that nicotinic antagonists were potential candidates as analgesics in the periphery. We decided to investigate the possibility that less-studied marine species, including mollusc-hunting and worm-hunting cone shells, might possess compounds with high selectivity towards *neuronal-type* nicotinic receptors.

1.1. Marine sources of nicotinic acetylcholine receptor antagonists

(i) The Japanese Ivory Mollusc, *Babylonia japonica* (neosurugatoxin) (Fig. 1):

The death of several individuals from ingestion of the Japanese Ivory mollusc *Babylonia japonica* led to identification of surugatoxin, a potent nicotinic receptor antagonist (Kosuge et al., 1981). Further examination showed that the source of the toxin was a marine bacterium, which inhabited the midgut gland. An even more potent toxin, neosurugatoxin (NSTX), a heterocyclic glycoside with a pentacyclic aglycone, was identified by Kosuge and colleagues. A sample of this was obtained by our laboratory and tested on primary monolayers of bovine adrenal chromaffin cells, a neuronal-nicotinic receptor preparation (Marley and Livett, 2004). NSTX was found to be a highly active and competitive inhibitor of the neuronal nicotinic response. NSTX inhibited acetylcholine- and nicotine-induced catecholamine secretion from the cultured cells with an IC_{50} against 5 μM nicotine of just 30 nM (Bourke et al., 1988; McGlashan and Livett, 1993). This inhibitory effect was reversible and independent of the presence of an agonist. Neosurugatoxin remains the most potent of the marine nicotinic receptor antagonists tested to date. Unfortunately, it is quite non-selective, inhibiting muscle-type nicotinic responses as well (Luetje et al., 1990; Hong et al., 1992). NSTX did not possess the selectivity we required for the development of new therapeutics towards the neuronal-type nicotinic receptor. For this reason we turned our attention to screening novel compounds from marine corals and from temperate marine sponges collected in the southern waters of Australia and the Great Australian Bight.

1.2. Marine corals (lophotoxins, pukalides) and sponges (luffarins and cometins) (Fig. 1)

Several active components were identified from Australian soft corals including lophotoxin and deoxylophotoxin, diterpenes from corals of the genus *Lophogorgoria* (Abramson et al., 1988), and two related diterpenes, pukalide and epoxypukalide, all of which inhibited the nicotinic response from bovine chromaffin cells with IC_{50}s in the low micromolar range (Livett et al., 1991).

This work then led BGL and JGD to embark on a collaborative study with Rob Capon in the Chemistry Department, University of Melbourne, to survey extracts from Australian marine sponges for their ability to inhibit the neuronal-type nicotinic receptor responses in chromaffin cells. Positive hits were scored with two families of sesterterpenes, the luffarins (Dean et al., 1994) and cometins (Down et al., 1995; Broxton et al., 1996). Numerous compounds were investigated in functional assays for both muscle-type and neuronal-type nAChRs. With neuronal nAChR activities as potent as IC_{50} 15 μM, these compounds are amongst the most active of the nitrogen-free nicotinic antagonists (Legg et al., 1997). But once again these lacked specificity in that they could not distinguish between muscle-type and neuronal-type nAChRs or between the different subunit combination variants of neuronal-type nicotinic receptors.

1.3. Cone snails (conotoxins)

The lack of tissue and subunit specificity of the above compounds led us to examine α-conotoxins, first in Australian fish-eating species (*C. geographus, C. striatus* and *C. magus*) confirming their muscle-type nAChR specificity, and then in Australian molluscivorous (*C. imperialis, C. textile* (see cover image), *C. episcopatus*, *C. victoriae* (Fig. 2a,b)) and

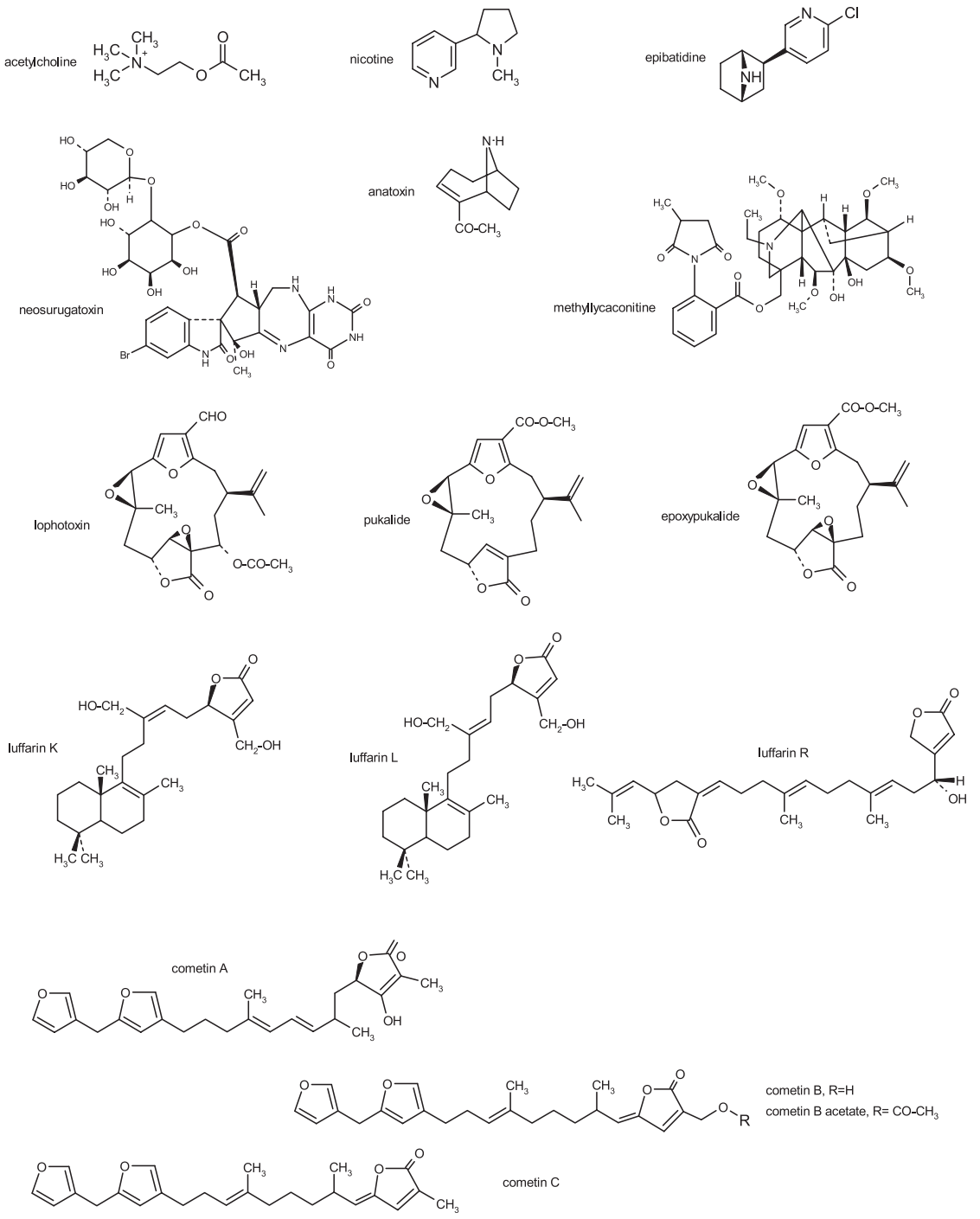


Fig. 1. Non-peptide nicotinic receptor agonists and antagonists.

vermivorous (*C. pennaceus, C. anemone, C. leopardus,*) cone snails for the presence of neuronal-type nicotinic receptor antagonists. Interest in the potential analgesic properties of *Conus* venom components was triggered by early observations that victims of cone snail envenomation died "a painless death" (Cleland, 1912; Flecker, 1936; Fegan and Andresen, 1997).

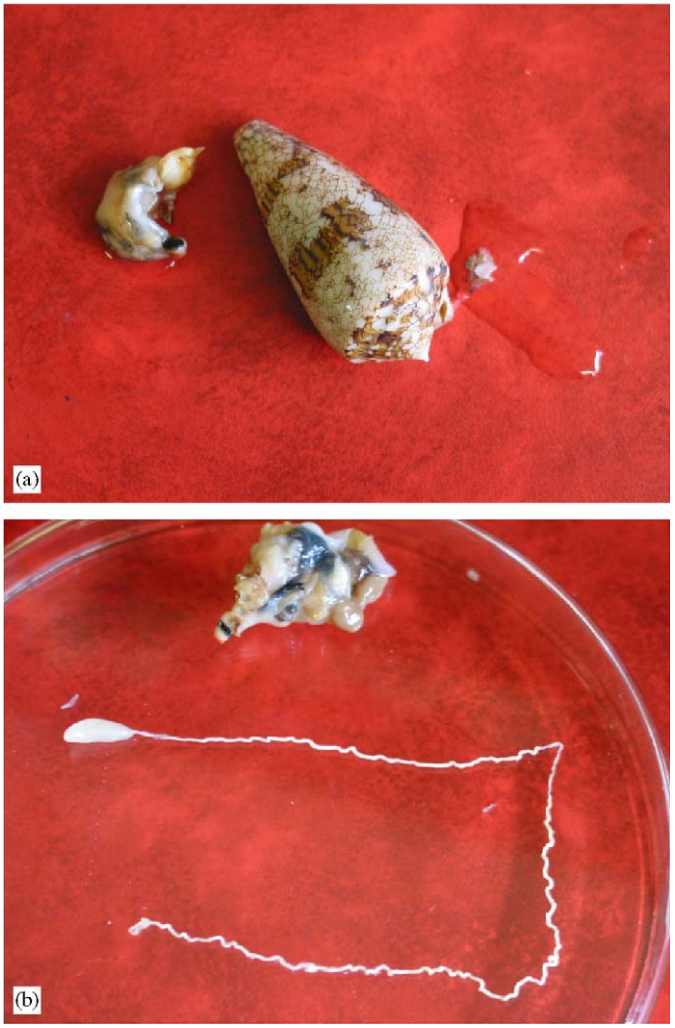


Fig. 2. (a) *Conus victoriae* with animal removed prior to dissection of venom duct. (b) Venom duct dissected from *Conus victoriae* showing animal removed.

1.3.1. Early observations on cone shell envenomation in Australia

Reports of instances of fatal poisoning by the various species of cone snail, *Conus*, were so rare that up to 1936 no such case occurring in Australian waters had been recorded (Flecker, 1936). Curare-like symptoms were noted from as early as 1901 when Hallen described a case in Fiji of slow paralysis caused by *C. geographus* envenomation (see Cleland, 1912). The first case in Australia was reported in 1935 and involved envenomation by *C. geographus* at Haymen Island. The symptoms were recorded by a medical practitioner who reported, "the sight became blurred with diplopia; at thirty minutes the legs were paralysed; and at sixty minutes unconsciousness appeared and deepened into coma.... Just before death, the pulse became weak and rapid, with slow, shallow respirations. Death took place five hours after the patient was stung. The symptoms resemble much those of *curare* poisoning as described in earlier reports..." (Flecker, 1936).

It is now known that these curare-like effects of *C. geographus* venom are exerted by conotoxin GI which acts to block nicotinic acetylcholine receptors at the neuromuscular junction (Spence et al., 1977; Olivera et al., 1990). This causes the diaphragm muscle to fail resulting in asphyxiation of the victim

Although antagonists of the nicotinic acetylcholine receptor from the venom of these fish-hunting species (Duda and Palumbi, 2004) were quite early

suspected as being the curare-like components responsible for asphyxiation and death of the victim, the presence of conotoxins in non-piscivorous species exhibiting antagonism towards *neuronal* nicotinic receptors antagonists was not recognized until the mid 1990s (Fainzilber et al., 1994; McIntosh et al., 1994). Likewise the potential of neuronal specific alpha conotoxins as analgesics remained unexplored.

1.4. Studies with non-piscivorous Conus species as sources of toxins targeting the neuronal-type nicotinic receptor

1.4.1. Conus imperialis

In a collaborative venture with the University of Queensland we showed that α-conotoxin ImI from the venom of the vermivore *C. imperialis* (collected from Lady Elliott Island) was a selective inhibitor of the mammalian neuronal nAChR in chromaffin cells. α-conotoxin ImI did not inhibit the muscle-type nicotinic receptor response in the rat phrenic-nerve hemidiaphragm preparation (Broxton et al., 1999). These studies provided proof of concept that α-conotoxin ImI could target mammalian neuronal nAChRs while not affecting muscle-type nAChRs, as suggested from earlier studies in which ImI was shown to induce seizures when injected into the brain of mice and rats (McIntosh et al., 1994). This conotoxin is the smallest of the α-conotoxins discovered having only 12 amino acids with two disulfide bonds in an unusual 2–8, 3–12 linked construct, a so-called 4:3 loop structure.

1.4.2. Conus pennaceus

In studies on *C. pennaceus* the selectivity of two α-conotoxins PnIA and PnIB were examined for their effect upon the neuronal nicotinic response. These two conotoxins which differ only in positions 10 and 11 (Table 2; Cartier et al., 1996; Dowell et al., 2003; Gray et al., 1981; Lu et al., 1999; Luo et al., 1998; McIntosh et al., 2002, 2005; Olivera et al., 2000; Teichert et al., 2004) were found to differ significantly in their ability to inhibit the neuronal nicotinic response. These toxins had previously been shown to block neuronal ACh receptors in molluscs (Fainzilber et al., 1994). We showed that Leu^{10} of α-conotoxin PnIB confers potency for neuronal nicotinic responses (Broxton et al., 2000). The importance of this amino acid position in determining selectivity towards the neuronal-type nicotinic receptors was confirmed in two other studies (Hogg et al., 1999; Luo et al., 1999).

1.4.3. Conus episcopatus

A novel tyrosine sulfated conotoxin, α-conotoxin EpI was identified in the venom of *C. episcopatus,* a molluscivore we collected from Heron Island. Both this toxin and its non-sulfated analogue were tested using the neuronal nicotinic receptor assay on bovine adrenal chromaffin cells. Both peptides were found to be potent inhibitors of the neuronal nAChR response (Loughnan et al., 1998).

1.5. Venom limiting approach

The traditional approach of collecting duct venom and fractionating it by HPLC to obtain the individual conotoxins posed problems in terms of the number of snails required for a complete isolation and characterization of a novel conotoxin. In addition, restrictions imposed by the Great Barrier Reef Marine Park Authority on the number of cone snails of any one species that could be collected to a maximum of 5 specimens, prompted us to adopt a cDNA approach as a more conservative way of discovering novel conotoxins.

Instead of isolating peptides for sequencing from duct venom our aim was to develop a DNA-based approach for conotoxin prospecting (Maksel et al., 1998; Gayler et al., 2005). PCR is a popular and sensitive technique for identifying rare-gene products (Maksel et al., 1998). Poorly expressed transcripts can be identified in the cDNA; even when the corresponding peptide cannot be detected or isolated from the venom. The high sensitivity and amplification afforded by this cDNA approach allowed us to obtain the conotoxin gene sequence information required from as little as one specimen of a given *Conus* species. To achieve this objective we employed rapid amplification of cDNA ends (RACE) by PCR. PCR-RACE made it possible to amplify conotoxin transcripts from a particular superfamily using a single primer with the AP1 adaptor. This is possible because transcripts encoding peptides of each superfamily of conotoxins are known to exhibit a conserved region at the 5′ end (Fig. 3). For the A-superfamily, this conserved region of DNA encodes the very distinctive methionine-rich sequence (MGMMR..) at the amino-terminal end of the precursor peptide (Table 2).

This was identified from α-conotoxins available in the NCBI database. Sequence identity is also high in

Table 2
Comparison of amino acid sequences of the signal sequence, pre- and mature toxin regions of some conotoxins

Conotoxin	*Conus* species	Signal Sequence Region	Pro-Region	Predicted or *Actual Mature Toxin	Reference
A-Superfamily					
(α4/7) α-conotoxins					
molluscivorous					
AulA	*C. aulicus*			GCCSYPPCFATNSDYC-NH2	Luo et al. (1998)
AulC	*C. aulicus*			GCCSYPPCFATNSGYC-NH2	Luo et al. (1998)
AulB	*C. aulicus*			GCCSYPPCFATNPD-C-NH2	Luo et al. (1998)
Bn1.1	*C. bandanus*	MGMRMMFTMFLLVVLATTVVS	FASDRASDGRNAAAKDKA	GCCSHPACSVNNPDIC-NH2	Santos et al. (2004)
Bn1.2	*C. bandanus*			ECCTHPACHVSHPELC-NH2	Santos et al. (2004)
Ca1.2	*C. Caracteristicus*	MGMRMMFTVFLLVVLATTVVS	FTSDRASEGRNAAAKDKA	GCCAIRECRLQNAAYCGGIY	Santos et al. (2004)
Da1.1	*C. dalli*	MGMRMMFTVFLLVVLATTVVS		GCCSHPACNVDHPEIC-NH2	Olivera et al. (2002)
EpI*	*C. episcopatus*			GCCSDPRCNMNNPDYC-NH2	Loughnan et al. (1998)
PnIB*	*C. pennaceus*			GCCSLPPCALSNPDYC-NH2	Fainzilber et al. (1994)
PnIA*	*C. pennaceus*			GCCSLPPCAANNPDYC-NH2	Fainzilber et al. (1994)
Vc1.1	*C. victoriae*	MGMRMMFTVFLLVVLATTVVS	STSGRREFRGRNAAAKA	SDLVSLTDKKRGCCSDPRCNYDHPEIC-NH2	Sandall et al. (2003)
Vc1a*	*C. victoriae*			GCCSDORCNYDHPγIC-NH2	Jakubowski et al. (2004)
piscivorous					
GIC	*C. geographus*		SDGRNDAA__KA	FDLISSTVKKGCCSHPACAGNNQHIC-NH2	McIntosh et al. (2002)
MII*	*C. magus*	MGMRMMFTVFLLVVLATTVVS	FPSDRASDGRNAAANDKA	SDVITLAL-KGCCSNPVCHLEHSNLC-NH2	Cartier et al. (1996)

PeIA	*C. pergrandis*		GCCSHPACSVNHPELC-NH2	McIntosh et al. (2005)
PIA	*C. purpurascens*		RDPCCSNPVCTVHNPQIC-NH2	Dowell et al. (2003)
Txα1	*C. textile*	MGMRMMFIVFLLVVLATTVVS	PECCSDPRCNSSHPELCG-NH2	Lu et al. (1999)
Txα2	*C. textile*	MGMRMMFTVFLLVVLATTVVS	PECCSHPACNVDHPEICR	Lu et al. (1999)
vermivorous				
An1.1	*C. anemone*	MGMRMMFIMFLLVVLATTVDSITSDRASLARKAAADLVALTVREGCCSHPACYANNQDYC-NH2		Sandall et al. (2002)
AnI-A*	*C. anemone*		CCSHPACYANNQDYC-NH2	Loughnan et al. (2004)
ImI (α4/3)	*C. imperialis*		GCCSDPRC-A--W-RC-NH2	McIntosh et al. (1994)
Lp1.1 (α3/5)	*C. leopardus*	MGMRMMFTVFLLVVLATTVVSFPSERASDGRDDTAKDEGSDMEKLVEKKECCNPACGRHYSC-NH2		Gray et al.(1981)
GIB (α3/5)	*C. geographus*	MGMRMMFTVFLLVVLATTVVSFPSERASDGRDDTAKDEGSDMEKLVEKKECCNPACGRHYSC-NH2		Santos et al.(2004)
SI* (α3/5)	*C. striatus*	MGMRMMFTVFLLVVLATTVVSFPSDRASDGRDDEAKDDERSDMHESDRKEICCNPACGPKYSC-NH2		Zafaralla et al.(1988), Lu et al.(1999)
SII* (α3/5)	*C. striatus*	MGMRMMFTVFLLVVLATTVVSFPSDRASDGRDDEAKDERGCCCNPACGPNYGCGTSCS		Ramilo et al.(1992), Santos et al.(2004)
Some other αA-conotoxins				
Bn1.3 (α4/3)	*C. bandanus*	MGMRMMFTVFLLVVLATAVLP	DYCCHRGPCMVWC-NH2	Santos et al.(2004)
S1.1 (α4/4)	*C. striatus*	MGMRMMFTVFLLVVLAITVVS	NGCCRNPACESHRC-NH2	Santos et al.(2004)
Ca1.1 (α4/5)	*C. caracteristicus*	MGMRMMFTVFLLVVLATTVVS	ZNCCSIPSCWEKYKCS	Santos et al.(2004)
OIVA*(6/2:1/3)	*C. obscurus*		CCGVONAACHOCVCKNTC-NH2	Teichert et al.(2004)

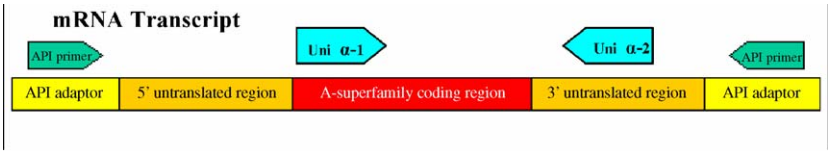
Table 2 (*continued*)

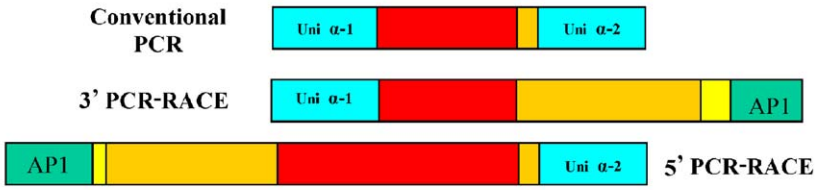
Conotoxin	*Conus* species	Signal Sequence Region	Pro-Region	Predicted or *Actual Mature Toxin	Reference
O-superfamily					
ω-conotoxins					
GVIA	*C. geographus*	MKLTCVVIVAVLLLTA	CQLITADDSRGTQKHRALGSTTELSLS	TRCKSPGSSCSPTSYNCC_RSCNPYTKRCYG	Cruz et al.(1978)
MVIIA	*C. magus*	MKLTCLVIVAVLLLTA	CQLLTADDSRGTQKHRALRSTTKLSNS	TRCKGKGAKCSRLMYDCCTGSCR__SGKCG	
CVID	*C. catus*	MKLTCLVIVAVLLLTA	CQLITANDSRGTQKHRALRSDTKLSMS	TRCKSKGAKCSKLMYDCCSGSCT__VGKCG	
κ-conotoxins					
MVIII	*C. magus*	MGMRMMFTVFLLVVLA	TTVVSIPSDRASDGRNAVVHERAPELVVTA	TTNCCGYNPMTICPPCMCTYSCPPKRKPGRRN	
SVIII	*C. striatus*	MGMRMMFTVFLLVVLA	TNVVSTPSDRASDGRNAAVHERQKSLVPSVI	TTCCGYDPGTMCPPCRCTNSCG	
δ-conotoxins					
TxVIA	*C .textile*	MKLTCMMIVAVLFLTAWTFATA	DDPRNGLGNLFSNAHHEMKNPEASKLN	KRWCKQSGEMCNLLDQNCCDGYCIVLVCT	Fainzilber et al. (1991)
PVIA	*C. purpurascens*	MKLTCVMIVAVLFLTAWTFVTA	DDSKNGLENHFWKARDEMKNREASKLD	KKEACYAPGTFCGIKPGLCCSEFCLPGVCFGG	Shon et al. (1995)

Y indicates sulfated tyrosine. Conserved cysteines that form the disulfide-bond framework are in bold. Underlined pairs of residues represent the cleavage boundary of the prepro region and the mature toxin peptide

Note:

1. Similarities in propeptide sequences between the α-conotoxins within the α3/5 series (muscle-type nicotinic receptor antagonists, e.g. GIB, SI and SII) and the differences between this series and the α4/7 (neuronal nicotinic receptor antagonists, e.g. GIC, MII, EpI and Vc1.1). These two groups of α-conotoxins are more similar to each other (both being α-A conotoxins) than to other conotoxins within the A-superfamily such as the αA-conotoxins. For an extensive presentation and of the structural and functional divergence of the A-Superfamily (see Santos et al., 2004).
2. Hypervariability in sequence characteristic of the functional mature conotoxin peptides is *not* seen in the pre and pro segments of the precursors. In contrast the sequences within the pre and pro regions of the precursors show a high degree of conservation when grouped according to the functional classification of their toxin region. For example, comparing the precursor sequence of ω-conotoxins GVIA and MVIIA, both of which block the N-type voltage-gated calcium channel and compete with each other for binding, it is notable that *the signal sequences (pre-region) are entirely identical.*
3. In addition there are conserved pre- regions among other classes of conotoxins (e.g. α, δ, κ conotoxins). Sequence identity is also high in the N-terminus of the pro-region and in the middle of the pro and around the cleavage site of the pro with the peptide. Not only are the pre- and pro- segments conserved, but the conotoxins cleaved from within the pre-pro-peptides are associated with particular functional classes.





Primer	Sequence (5'⟶ 3')
Uni-α1	ATGGGCATGCGGATGATGTT
Uni-α2	CGGAAAGTGAAGCAGGTCAG

Fig. 3. The diagram shows the binding site of the primers used. The sequence of the forward primer, Uni-α1 and of the universal reverse primer Uni-α2 is given.

the N-terminus, middle, and adjacent to the cleavage site of the pro-region of the peptide (Table 2).

A second conserved sequence in the 3′ untranslated region of α-conotoxins was observed. This provided a second anchor point to prime PCR reactions, enabling α-conotoxins to be amplified with paired gene-specific primers. The second primer site enhanced the specificity and selectivity of PCR from the mixed cDNA population.

Our study commenced with *C. anemone* (Sandall et al., 2002b), a small vermivorous species found in the temperate waters of southern Australia. PCR-RACE technology was utilized to amplify mRNA coding for conopeptides. Seven other species of *Conus* were studied (*C. victoriae, C. virgo*, *C. leopardus*, *C. marmoreus*, *C. textile*, *C. arenatus* and *C. magus*), encompassing the three main feeding classes, herbivores, molluscivores and piscivores (Sandall et al., 2002a, b). Up to 5 specimens of each species were collected and analysed in this study, from locations shown below.

Species	*Feeding type*	*Collected from*
C. anemone	Vermivorous	Edithburgh, South Australia
C. arenatus	Vermivorous	One Tree Island, GBR, Queensland
C. virgo	vermivorous	One Tree Island, GBR, Queensland
C. leopardus	Vermivorous	Heron Island, GBR, Queensland
C. marmoreus	Molluscivorous	Lizard Island, GBR, Queensland
C. textile	Molluscivorous	One Tree Island, GBR, Queensland
C. victoriae	Molluscivorous	Broome, Western Australia
C. magus	Piscivorous	Lizard Island, GBR, Queensland

2. The DNA-based approach

mRNA was isolated from frozen pulverized venom duct (the venom bulb being included to provide carrier RNA), via magnetic bead separation using Dynabead technology (Sandall et al., 2003). Reverse transcription produced double-stranded cDNAs, which had adaptor sites ligated to each end. PCR and PCR-RACE were then used to identify specific classes of conotoxins by isolating and sequencing the transcripts that encode them. We focussed on the A-superfamily of conotoxins (which includes the α-conotoxins, αA- and κ-A-conotoxins). PCR reactions were primed with one of three primer combinations. These were based on two gene specific primers (uni-α1 and uni-α2, Fig. 3), and the adaptor primer (AP1) that is universal for all of these cDNAs. This provided three separate PCR reactions that could be performed on each cDNA preparation; a traditional PCR using the two gene specific primers, a 3′ RACE PCR using uni-α1

and AP1, or a 5′ RACE PCR using uni-α2 and AP1. By combining all three PCR reactions, a complete cDNA transcript could often be amplified and sequenced (Fig. 3).

The DNA approach identified large numbers of divergent conotoxin sequences. The transcripts varied in sequence, length and even the number of cysteine residues responsible for forming the disulfide backbone. For example, an A-superfamily conotoxin (termed M4.1) was identified from *C. magus* with almost identical sequence to κ-A conotoxins MIVA (Santos et al., 2004) and SIVA from *C. striatus*, both known antagonists of voltage-gated K^+ channels (Craig et al., 1999).

Use of the PCR approach also revealed variation in expression of conotoxins between individual specimens of the same *Conus* species. In an earlier report, Bingham et al. (1996) had reported variation in the conotoxin components of the venom from individual specimens of *C. geographus*. Our studies revealed variation in conotoxin expression was common for all species. For example, of four α-conotoxins from *C. leopardus*, two were expressed in one specimen (Lp1.1. and Lp1.2) and three were expressed in the other (Lp1.2, Lp1.3 and Lp1.4). It is likely that in any one specimen only a subset of conotoxins is ever expressed from the genome. The implications of this conclusion are that when searching for novel conotoxins from a given species it is prudent to search the mRNA extracted from a number of specimens. The combination of multiple venom ducts was particularly useful in the case of *C. anemone* where eight separate transcripts encoding α-conotoxins were identified (Sandall et al., 2002a, b).

Examination of the amino acid sequences of 28 known α-conotoxins from 14 different species (5 piscivores, 6 molluscivores and 3 vermivores) had revealed two consensus sequences (shown below), one characteristic of α-conotoxins (3:5 loop) active at muscle-type nAChRs and the other of α-conotoxins (4–7 loop) active at neuronal-type nAChRs.

2.1. Generic sequence

(Where X is any amino acid (present or absent) and C* is a C-terminal amide)

Muscle type	**C C** N/H P A **C** G R/K N/H Y/F X **C***	[3:5 loop]
Neuronal type	G **C C** S X P X **C** X X X N/H P X X **C***	[4:7 loop]

These features provided a basis for predicting the muscle-type or neuronal-type nAChR activity of any new α-conotoxin based on its primary structure.

2.2. Application of the cDNA approach to the discovery of ACV1: an α-conotoxin from C. victoriae with therapeutic potential

2.2.1. Selection of the sequence to be synthesised

Twenty novel α-conotoxin sequences were discovered from the 8 species of *Conus* studied, and 10 of these were synthesized based on best match to the generic sequence for neuronal-type α-conotoxins. Attention was given to the deduced peptide sequence Vc1.1 from *C. victoriae* as it had the highest similarity to the α-conotoxins EpI, MII, PnIA and PnIB, which we and others had shown to be antagonists of the neuronal nicotinic receptor (Luetje et al., 1990; Fainzilber et al., 1994; Loughnan et al., 1998; Broxton et al., 2000). The candidate conotoxin Vc1.1, of 16 amino acids with a C-terminal amide, contained four cysteinyl residues in a CC–C–C arrangement characteristic of the α-conotoxins. Moreover, it had the 4:7 loop structure characteristic of conotoxins that inhibit the neuronal nicotinic receptor. This putative α-conotoxin was subsequently shown pharmacologically to be an α-toxin (see below) and so was termed "α-conotoxin Vc1.1".

2.2.2. In-vitro pharmacological tests

The α-conotoxins, to which Vc1.1 belongs, are antagonists of nicotinic acetylcholine receptors. Peripheral sensory nerves and dorsal root ganglion cells involved in pain transmission contain neuronal nicotinic receptors (Genzen et al., 2001; Lang et al., 2003). Being antagonists of the neuronal nicotinic receptors the α-conotoxins were of interest to us as potential analgesics. The potential of Vc1.1 as an analgesic was dependent on whether it was specific for the neuronal subtype of the receptor. Accordingly we tested Vc1.1 against two classical muscle preparations, the phrenic nerve-hemidiaphragm preparation and the chick *biventer cervicis* preparation. In accord with the predicted 4:7 loop classification of Vc1.1 as a *neuronal*-type nicotinic receptor antagonist, it was without effect on the two muscle nAChR preparations at concentrations up to 50 μM. When Vc1.1 was assayed for activity in a functional *neuronal* nicotinic receptor assay, monolayer cultures of bovine adrenal chromaffin cells it acted, as predicted, as a competitive inhibitor with

an IC_{50} of 1–3 μM. Vc1.1 also competed with the nicotinic agonist [^{3}H-epibatidine] for binding to preparations of bovine adrenomedullary cell membranes (Sandall et al., 2003). The particular subunit composition of the neuronal nicotinic receptor to which Vc1.1 interacts in bovine chromaffin cells is not known but is likely to be a heterotrimeric $\alpha3\beta4$ receptor containing $\alpha5$ and/or $\alpha7$ subunits. Both $\alpha5$ and $\alpha7$ nAChR subunit mRNAs have also been detected in human sensory nerves (Genzen et al., 2001; Lang et al., 2003). We therefore decided to test Vc1.1 for its effect on sensory nerve function.

2.2.3. *Vc1.1 inhibited a vascular response to sensory nerve function*

Most tests of sensory nerve function involve long-term experiments to measure pain responses in experimental animals in vivo. These are not suited to rapid screening. However, one test that provides a relatively rapid indication of C-fibre function and which is non-invasive is to measure the vascular response to selective nerve stimulation of sensory nerves (Khalil and Helme, 1989). Vc1.1 was shown to suppress the vascular response to unmyelinated sensory nerve C-fibre activation in rats (Satkunanathan et al., 2002; Sandall et al., 2003). Furthermore, its ability to suppress C-fibre function was greater than that of MVIIA, an ω-conotoxin with known analgesic activity in rats (Wang et al., 2000) and humans (Staats et al., 2004). While this technique does not measure pain directly, it provided an indirect measure of C-fibre activity. The results indicated a potential for Vc1.1 to suppress pain in animals and in humans. Accordingly, we investigated the ability of Vc1.1 to suppress the response to mechanical hyperalgesia in two animal models of peripheral neuropathic pain. Such vascular responses are representative of C-fibre activity involved in pain transmission.

2.2.4. *Vc1.1 attenuated the pain response in two animal models of peripheral neuropathy*

Vc1.1 was next tested in two models of peripheral neuropathy of the rat sciatic nerve, the chronic constriction injury (CCI) model of Bennett and Xie (1988) and the partial nerve ligation (PNL) model of Seltzer et al. (1990). The CCI model mimics the neuropathy or spinal root irritation due to lumbar disk herniation. The PNL model mimics the clinical condition of an accidental nerve bruise or gunshot-induced nerve injury (see Zimmerman, 2001). In both these models Vc1.1 significantly attenuated the mechanical hyperalgesia. The data obtained from a direct measure of pain behaviour in two animal models of human neuropathic pain provided direct evidence that Vc1.1 is effective in suppressing neuropathic pain of peripheral origin (Satkunanathan et al., 2005). Vc1.1 had no effect on mechanical analgesia threshold in uninjured animals. It is likely therefore that Vc1.1 inhibits C-fibre activity by blocking neuronal nAChRs located on injured C-fibres. Vc1.1 has also been shown to be an effective analgesic in the partial ligation model that mirrors traumatic back pain injuries (Satkunanathan et al., 2004).

A recent study has identified neuronal-type nAChR subunits $\alpha3$, $\alpha5$ and $\beta4$ as the principal subtypes present in human sural nerve, a peripheral sensory nerve (Lang et al., 2003, 2005). By blocking such nAChRs, Vc1.1 could bring about a reduction in sodium and calcium influx and a reduction in sensitisation of the voltage gated calcium channels with a consequent reduction of ectopic discharge from sensory neurons. This is one mechanism by which Vc1.1 could bring about attenuation of hyperalgesia. The exact receptor subtype combination that ACV1 binds to is yet to be determined.

2.2.5. *Vc1.1 accelerated the rate of recovery from a nerve injury*

In the process of evaluating Vc1.1 as an analgesic in the CCI model above, Dr. Zeinab Khalil made an important additional observation; Vc1.1 had the ability to accelerate the rate of recovery from a nerve injury. Although the mechanism involved is not known, it is unlikely to be via inhibition of the neuronal nicotinic receptors since an analogue of Vc1.1, lacking both nicotinic receptor activity and lacking analgesic activity, nevertheless retained the ability to accelerate recovery from a nerve injury (Satkunanathan et al., 2005).

This particular analogue of Vc1.1 with hydroxyproline in place of proline and gamma-carboxy glutamate in place of glutamate was synthesised by solid phase peptide synthesis. A peptide (vc1a) with this modified sequence was subsequently identified by mass spectroscopy in the duct venom of *C. victoriae* (Jakubowski et al., 2004). However, as mentioned above, the synthetic modified peptide lacked antagonism against the nicotinic receptor and was not an analgesic in the CCI pain model (Livett et al., 2002). This suggests that the ability of Vc1.1 to act as an analgesic was related to its ability to inhibit the neuronal nicotinic receptor whereas its

Table 3
Conopeptides being developed for the treatment of neurological conditions

Name	Conopeptide	*Conus* species[a]	Target	Stage	Company[b]	Comment	Reference
ACV1	α-Conotoxin Vc1.1	*C. victoriae (m)*	Competitive blocker of selected *neuronal-type* nicotinic ACh receptors	Phase II	Metabolic Pharmaceuticals Ltd, Melbourne, Vic, Australia	Effective against peripheral neuropathic pain in animal models and accelerates functional recovery of injured neurons	Sandall et al. (2003), Livett et al. (2002), Livett et al. (2004),Gayler et al. (2005), Satkunanathan et al. (2005)
	rho- Conotoxin TIA	*C. tulipa (p)*	Reversible non-competitive inhibitor of α-1 adrenergic receptors	Preclinical	Xenome, Ltd., Brisbane, Qld., Australia	Acts as a reversible non-competitive inhibitor of alpha-1 adrenergic receptors	Sharpe et al. (2003b)
AM336	ω-conotoxin CVID	*C. catus (p)*	Blocks N-type calcium channel, Ca ((v)2.2) calcium channel variant.	Phase II	AMRAD Corp.—under license from University of Queensland	Being developed for neuropathic pain. Reported to have a better therapeutic index than Prialt™	Adams et al. (2003)
SNX-III, C1002, Ziconotide, Prialt™	ω-conotoxin MVIIA	*C. magus (p)*	N-type calcium channels	Completed Phase III, Approved by FDA 28/12/'04 and CHMP 18/11/'04 for the treatment of severe, chronic pain in patients who require intrathecal (IT) analgesia.	Elan Corporation (Elan Pharmaceuticals), CA, USA	Significant pain relief to patients in clinical trials.	Bowersox and Luther (1998), Heading (2001), Penn and Paice (2000), Levin et al. (2002), Atanassoff et al. (2000), Jain (2000)
						Side-effects in some patients. Hence call for repeat of Phase III clinical trials for cancer pain.	Valentino et al. (1993), Heading (2002), Azimi-zonooz et al. (2001)
						Also trialled (as C1002) for neuroprotection in ischemic stroke and	

						coronary bypass surgery but results were disappointing and trials have been abandoned.	
Xen2174	χ-conopeptides (chi-CTX MrIA/B)	*C. marmoreus (m)*	Acts as reversible noncompetitive inhibitor of the neuronal noradrenaline transporter	Phase I/II	Xenome, Ltd., Brisbane, Qld., Australia	Being developed to "treat certain types of pain, for which there is currently a lack of effective treatment" neuropathic pain.	Sharpe et al. (2003a)
CGX-1160	Contulakin-G	*C. geographus (p)*	Binds to neurotensin receptor	Phase II	Cognetix Inc, Salt Lake City, USA	Short term management of post-operative pain	Malmberg et al. (2003)
CGX-1007	Conantokin-G	*C. geographus (p)*	Selective inhibitor of the NMDA receptor (NR2B subtype)	Phase II	Cognetix Inc, Salt Lake City, USA	Potent antinociceptive effects in several models of injury-induced pain. Also, control of seizures in intractable epilepsy	Malmberg et al. (2003)
CGX-	Conantokin-T	*C. tulipa (p)*	Selective inhibitor of the NMDA receptor (NR2A and NR2B) subtypes	Phase II	Cognetix Inc, Salt Lake City, USA	Potent antinociceptive effects in several models of injury-induced pain	Malmberg et al. (2003)

[a]Prey preference for *Conus* species: p = piscivorous (fish-hunting); *m* = molluscivorous (mollusc hunting); *v* = vermivorous (worm-hunting).Note: This list does not attempt to be comprehensive. For other examples of *Conus* peptides being investigated for therapeutic potential see the excellent reviews by Alewood et al., 2003; Heading, 2001, 2002; Doggrell, 2004; Grant et al., 2004).

[b]Websites of commercial developers: Elan Corporation http://www.elan.com ; Cognetix Inc. http://www.cognetix.com ; Xenome Pty Ltd: http://www.xenome.com ; Amrad Corp. http://www.amrad.com.au ; Univ. of Melbourne: Cone Shell and Conotoxin Homepage http://grimwade.biochem.unimelb.edu.au/cone/ ; Metabolic Pharmaceuticals Ltd.: http://www.metabolic.com.au.

ability to accelerate the rate of recovery from a nerve injury was not.

2.2.6. Further studies complementing the action of Vc1.1's action as an analgesic

Independent testing of conotoxin Vc1.1 by Metabolic Pharmaceuticals Ltd., Melbourne, (termed ACV1 for commercial development) has confirmed its analgesic activity in two animal models of neuropathic pain. Both mechanical and tactile analgesia were effectively suppressed. The peptide is also effective in an acute pain model involving administration of capsaicin to the conjunctiva in the rat and acute pain induced by application of substance P to the eye (unpublished). One of the advantages of ACV1 is its excellent tolerability, well above the therapeutic range and its effectiveness when given by subcutaneous injection, a convenient route for administration. In October 2003, a licensing agreement was signed with Metabolic Pharmaceuticals Ltd. to develop ACV1 as an analgesic for a range of painful neurological conditions. ACV1 is currently undergoing Phase I clinical trials, having completed formal pre-clinical toxicity studies. Metabolic Pharmaceuticals believes that ACV1 will have wide applicability for many types of chronic pain including diabetic neuropathy and shingles, for which conventional analgesics offer little relief.

Of particular interest is a recent finding (Lang et al., 2005), that ACV1 is effective in inhibiting nicotine-induced increases in excitability of unmyelinated C-fibre axons in isolated segments of peripheral human nerves (obtained after amputation or nerve biopsy). ACV1 also blocked the increased excitability induced by acetylcholine. In these experiments in vitro, excitability of the unmyelinated axons was tested by threshold tracking, an extracellular electrophysiological recording technique using the compound C-fibre action potential (Lang et al., 2003). Furthermore, the antagonistic effect of ACV1 against acetylcholine but not against nicotine was reversed after inhibition of acetylcholinesterase activity in nerve segments by neostigmine, indicating competition between ACV1 and acetylcholine for a common binding site on the nicotinic receptors expressed on these peripheral sensory human nerves. Since sensory C-fibres convey pain information to the brain, inhibition of excitability in these peripheral nerves by ACV1 could account for its action as an analgesic (see Jain, 2004; Lang et al., 2005).

Of the α-conotoxins studied to date only one, the 16-residue α-conotoxin Vc1.1, has been reported to exhibit analgesic properties and is the only α-conotoxin currently under development as a potential therapeutic. Current treatments for neuropathic pain are ineffective and/or not well tolerated. Morphine causes dependence and its use is associated with unwanted side effects including dysphoria and constipation. Other treatments include use of antidepressants and anticonvulsants that act via a number of apparently non-specific mechanisms to cause general CNS depression and other unwanted side-effects (Dickinson et al., 2003). As a result there has been considerable interest in using agents that act more specifically to modulate ion channels involved in pain transmission.

2.3. Other conopeptides under development as potential therapeutics

This area has been the subject of several recent reviews (Shen et al., 2000; Jones et al., 2001; Alewood et al., 2003; Alonso et al., 2003; Lewis and Garcia, 2003; Mari and Fields, 2003; Heading, 2004; Livett et al., 2004; Gayler et al., 2005).

2.3.1. ω-conotoxins

The first such agents studied were N-type calcium channel blockers and consequently ω-conotoxins have attracted attention as antinociceptives (Bowersox et al., 1996; see Heading, 1999, 2002 for review). However, in clinical trials N-type calcium channel blockers have produced a range of unwanted side-effects upon long-term usage in humans (Penn and Paice, 2000). ω-conotoxin MVIIA (Ziconotide, Prialt) is the most advanced of these (Staats et al., 2004) and has recently received FDA approval for use in cancer pain. Another ω-conotoxin, CVID from *C. catus* is claimed to have a better therapeutic window than MVIIA (Adams et al., 2003; Lewis et al., 2000) and AMRAD received initial encouraging responses from subjects enrolled in a Stage I/II clinical trial. With respect to use of omega conotoxin MVIIA and CVID to control chronic pain, Blake et al. (2003) caution that intrathecal injections of these drugs may compromise cardiovascular responses to changes in central blood volume. At the single doses studied in rabbits, there were significant differences between the responses to simulated haemorrhage after ω-conotoxin MVIIA or the α2-adrenoceptor agonist dexmedetomidine compared with ω-conotoxin CVID, with the

prolonged effect after MVIIA most likely to be of clinical significance (Table 3).

Other conopeptides that are being investigated for their therapeutic potential include selective antagonists of the glycine site of NMDA receptors and selective NMDA NR2B receptor antagonists such as the conantokins (Parsons, 2001; Malmberg et al., 2003), a new μ-conotoxin (SMIIIa) that targets tetrodotoxin-resistant sodium channels in sympathetic and sensory neurons (West et al., 2002; Keizer et al., 2003), and contulakins that target a neurotensin-like receptor (Malmberg et al., 2003). The Brisbane-based pharmaceutical company Xenome Ltd. has reported on several conotoxins that are currently in preclinical development including an inhibitor of the α-1 adrenoceptor, with therapeutic potential for the treatment of urinary incontinence caused by disorders such as benign prostatic hyperplasia. Among these, are a χ-conotoxin that targets the neuronal noradrenaline transporter (Sharpe et al., 2003a) and the ρ-conotoxins that act as reversible non-competitive inhibitors of G-protein-linked α-1 adrenergic receptors (Sharpe et al., 2001, 2003b).

3. Conclusions

Together there are over 500 species of *Conus* with a large number of venom components encompassing many pharmacological families per species. While we have concentrated on the α-conotoxins as receptor probes and therapeutic agents the potential of these peptides as drug leads is not confined to any one class or superfamily. The α-conotoxins represent only a tiny fraction of the total conotoxin peptides available for investigation. The exquisite specificity that conotoxins exhibit for particular receptors and ion-channels is a function of the all conopeptide classes present in *Conus* venom; a conservative estimate is that there are at least 25,000 different venom peptides across the species. With the call for increasing conservation of this precious resource (Chivian et al., 2003; Duda et al., 2004; Fainzilber, 2004) researchers have begun to milk cone snails for their venom. Dr. Jon-Paul Bingham has been successful in milking a number of cone snails kept in captivity (Nelson, 2004). The application of molecular techniques together with the high sensitivity of modern mass spectrometry (Jakubowski et al., 2004; Loughnan et al., 2004; Loughnan and Alewood, 2004; Jakubowski and Sweedler, 2004; Garrett et al., 2005) and MS techniques for unambiguous determination of disulfide connectivity (Bingham et al., 2005) has enabled identification of novel *Conus* peptides in as little as one cm. of venom duct from a single specimen and in milked venom samples. The venom of this one genus may be the richest natural library of pharmacologically active compounds. The potential to "therapeutically mine" such a resource appears limitless (Jones and Bulaj, 2000). Given the obvious potential of this natural library, the challenge is to "mine" the resources in an efficient and conservative manner.

In the relatively short period of 30 years since Bob Endean began his studies on the pharmacological activity of venomous cone shells on the Great Barrier Reef (Endean, 1964), great progress has been made in revealing the diversity in structure and actions of these selective marine neurotoxins. We are now at an exciting stage where several of these cone snail peptides are being developed as potential therapeutics for a range of neurological conditions. These include painful neuropathic pain conditions such as diabetic neuropathy, shingles and cancer pain where current treatments are inadequate or associated with undesirable side effects, and conditions such as post-surgical pain and certain forms of epilepsy where new approaches to treatment would be welcomed. Potential applications of conopeptides are expanding and their development as therapeutics over the next decade presents a challenge and commands our further attention.

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