ISSN 1672-9145 Acta Biochimica et Biophysica Sinica 2004, **36**(11): 713–723 **CN** 31-1940/Q

Minireview

Conus Peptides—A Rich Pharmaceutical Treasure

Cheng-Zhong WANG[1,2] and Cheng-Wu CHI[1,3]*

[1]*Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, the Chinese Academy of Sciences, Shanghai 200031, China;*
[2]*Institute of Neuroscience, the Chinese Academy of Sciences, Shanghai 200031, China;*
[3]*Institute of Protein Research, Tongji University, Shanghai 200092, China*

Abstract Marine predatory cone snails (genus *Conus*) with over 500 species represent what is arguably the largest single genus of marine animals alive today. All *Conus* are venomous and utilize a complex mixture of *Conus* peptides to capture their preys and for other biological purposes. Each component of *Conus* peptides selectively targets a specific subtype of ion channels, neurotransmitter receptors or transporters. Owing to their diversity, more than 50,000 distinct active peptides are theoretically estimated in *Conus* venoms. These diversified toxins are generally categorized into several superfamilies and/or families based on their characteristic arrangements of cysteine residues and pharmacological actions. Some mechanisms underlying the remarkable diversity of *Conus* peptides have been postulated: the distinctive gene structure, gene duplication and/or allelic selection, genus speciation, and sophisticated expression pattern and post-translational modification of these peptides. Due to their highly pharmacological potency and target selectivity, *Conus* peptides have attracted extensive attention with their potentials to be developed as new research tools in neuroscience field and as novel medications in clinic for pain, epilepsy and other neuropathic disorders. Several instructive lessons for our drug development could be also learnt from these neuropharmacological "expertises". *Conus* peptides comprise a rich resource for neuropharmacologists, and most of them await to be explored.

Key words *Conus* peptide; conotoxin; neuropathic disorder; pharmaceutical potency

The Biology of Cone Snails

The predatory cone snails (genus *Conus,* family Conidae) with over 500 species may comprise the largest single genus of marine animals living today. These species inhabit in tropical reef environments throughout the world. According to their prey preference, cone snails can be classified into three major groups: the piscivorous preying upon fish (e.g., *Conus striatus*, *C. geographus*), the molluscivorous eating mollusk (e.g., *C. textile*, *C. pennaceus*) and the vermivorous feeding upon *polychaete* annelids (e.g., *C. imperialis*, *C. vexillum*). All cone snails are venomous predators. Owing to the highly toxic peptides stored in their venoms, these predators with locomotory disadvantages can easily immobilize and capture their agile preys, as well as escape from and defend against their predators and possibly deter the competitors. About 30 cases of human envenomation by fish-hunting cone snails have been recorded so far, in some cases fatal.

Conus are excellent 'experts' in neuropharmacology. They generate a variety of toxins in the long convoluted ducts and store them in venom bulbs. Besides these two elements, the venom apparatus of Conidae comprises proboscis, pharynx, radular sac and teeth. The piscivorous cone snails have long harpoon-like tooth with its tip armed with a large blade on one side and a long posteriorly-directed harpoon on the other side [1]. During *Conus* prey capture, the proboscis armed with a harpoon-like radular tooth is abruptly ejected, and the prey instantly tethered and subsequently envenomated via injecting venom through the disposable hollow chitinous tooth. Thus far, the astonishing scene of *Conus* capturing the prey has been comprehensively demonstrated in the fish-eating cone snails. Based on the preying strategy, the piscivorous *Conus* can be subgrouped into two general classes: 'hook-

Received: August 6, 2004 Accepted: October 18, 2004

*Corresponding author: Tel, 86-21-54921165; Fax, 86-21-54921011; E-mail, chi@sunm.shcnc.ac.cn

and-line fishing' cone snails (e.g., *C. purpurascens*, *C. striatus* and *C. magus*) and 'net-fishing' cone snails (e.g., *C. geographus* and *C. tulipa*). These two kinds of strategies were clearly illuminated in the vivid cartoon by Olivera [2]. For *C. purpurascens* using the 'hook-and-line' strategy to capture fish, two distinct immobilization phases are subsequently elicited: the excitotoxic shock and the neuromuscular block. The former stage takes place instantaneously after venom injection and stuns the fish rapidly, while the latter lags and completely abolishes the muscle action potentials of its prey [2,3]. The complicated arrays of *Conus* venom underlying these two distinct phases are under extensive studies.

Classification and Nomenclature of *Conus* Peptides

Conus species have developed many distinct venoms as a survival strategy for feeding and defense. Their venoms contain a diverse mixture of biologically active peptides, mostly small and structurally constrained. During the 50 million years of evolution, *Conus* peptides have been optimized to target specific ion channels, cell-surface receptors and transporters with very high affinities and selectivities. Although more than 50,000 unique peptide sequences exist in *Conus* venom by estimate, these small peptides can be generally classified into two major categories: (1) those in the majority with disulfide bonds, referred to as conotoxins, and (2) those in the minority with a single or no disulfide bond. Furthermore, they may be broadly grouped into several 'superfamilies' and 'families'. Peptides within the same 'superfamily' share a characteristic cysteine arrangement and a highly conserved signal sequence in the precursors, and members within the same 'family' have in common the unique disulfide bonds and pharmacological activity [4]. Based on these principles, *Conus* peptides discovered up to now have been clustered into eleven superfamilies (Table 1): A, M, O, P, S, T, I, conopressin, conantokin, contryphan and contulakin. The superfamily may be subdivided into several families with distinct pharmacological activities (indicated within the following brackets): A-superfamily (α-, αA-,κA-, and ρ-conotoxins); M-superfamily (μ- and

Table 1 Conotoxin superfamilies

Superfamily	Cysteine arrangement	Family	Pharmacological activity	Example
A	CC-C-C	α	Competitive AChR antagonist	α-GI
	CCC-C-C-C	α	Competitive AChR antagonist	α-SII
	CC-C-C	ρ	α1 adrenoceptor antagonist	ρ-TIA
	CC-C-C-C-C	αA	Competitive AChR antagonist	αA-EIVA
	CC-C-C-C-C	κA	VGKC blocker	κA-SIVA
M	CC-C-C-CC	μ	VGSC blocker	μ-GIIIA
	CC-C-C-CC	ψ	Non-competitive AChR antagonist	ψ-PIIIE
O	C-C-CC-C-C	ω	VGCC blocker	ω-GVIA
	C-C-CC-C-C	κ	VGKC blocker	κ-PVIIA
	C-C-CC-C-C	δ	Delay the inactivation of VGSC	δ-TxVIA
	C-C-CC-C-C	μO	VGSC blocker	μO-MrVIB
	C-C-CC-C-C	χ	Pacemaker-channel blocker	χ-PnVIIA
P	C-C-C-C-C-C	Spastics	?	gm9a, tx9a
S	C-C-C-C-C-C-C-C	σ	5-HT_3 receptor antagonist	σ-GVIIIA
I	C-C-CC-CC-C-C	Excitatory	?	r11a, r11b
		κI	BK VGKC up-modulator	κI-BtXA
T	CC-CC	τ	Presynaptic Ca^{2+} channel blocker	ε-TxIX
		χ	Noradrenaline transporter inhibitor	χ-MrI
No assignment	C-C	Conopressins	Vasopressin receptor agonist (GPCR)	Conopressin-S
No assignment	C-C	Contryphans	?	Contryphan-R
No cysteine	linear	Conantokins	NMDA receptor antagonist	Conantokin-G
No cysteine	linear	Contulakins	Neurotessin receptor agonist	Contulakin-G

AChR, acetylcholine receptor; VGKC, voltage-gated potassium channel; VGSC, voltage-gated sodium channel; VGCC, voltage-gated calcium channel; 5-HT, 5-hydroxytryptamine; NMDA, *N*-methyl-*D*-aspartate; GPCR, G-protein coupled receptor.

ψ-conotoxins); O-superfamily (ω-, κ-, δ-, μO-, and χ-conotoxins); P-superfamily (spastic conotoxins); S-superfamily (σ-conotoxin); T-superfamily (τ- and χ-conotoxins) and I-superfamily (excitatory peptides) [4–6]. Some other superfamilies not assigned thus far include conopressins, conantokins, contryphans and contulakins, and all of them are either linear or with a single disulfide bond and belong to the non-conotoxin category.

As there is a great diversity in peptide superfamilies and families of cone snail toxins, a variety of *Conus* peptide targets have been identified, including many voltage-gated and ligand-gated ion channels, as well as G-protein-coupled receptors [5,7,8]. Conotoxins that target voltage-gated ion channels comprise those delaying the inactivation of sodium channels (e.g., δ-conotoxins) as well as the blockers specific for sodium, calcium, pacemaker and potassium channels (e.g., μ-, ω-, χ- and κA-conotoxins, respectively). Included in the *Conus* peptides targeting ligand-gated ion channels are antagonists of *N*-methyl-*D*-aspartate (NMDA) receptor (e.g., conantokins) and serotonin receptor (e.g., σ-conotoxins) as well as competitive and non-competitive antagonists of nicotinic acetylcholine (ACh) receptor (e.g., α- and ψ-conotoxins, respectively). While contulakins and conopressins are the agonists of G-protein-linked receptors of neurotensin and vasopressin, respectively, ρ-conotoxins act as antagonists of G-protein-coupled α1-adrenoceptor. Noteworthily, ω- and δ-conotoxins share the same cysteine arrangement, but their biological targets are different. Conversely, κ- and κA-conotoxins both target K^+ channels but possess different disulfide patterns. The unique selectivity of *Conus* peptides for different physiological targets can be attributed to their well-organized bridging structure, loop variability, and spatial arrangement of functional groups in three-dimensional structure. The structure-function relationships of *Conus* peptides have been comprehensively reviewed recently [9].

κA- and κ-family conotoxins inhibiting voltage-gated K^+ channels together with δ-family conotoxins delaying the inactivation of voltage-gated Na^+ channels elicit the initial stage (excitotoxic shock) of two distinct immobilization phases during *Conus* prey capture, while the lagging phase (neuromuscular block) characteristic of complete paralysis is ascribed to the synergetic effects of α-, αA- and ψ-conotoxins competitively or non-competitively inhibiting the binding of ACh to the post-synaptic nicotinic receptor at the neuromuscular junction together with ω-conotoxins acting as blockers of presynaptic voltage-gated Ca^{2+} channels to inhibit neurotransmitter release and μ-conotoxins like tetrodotoxin directly blocking skeletal muscle voltage-gated Na^+ channels [2,3]. These two non-overlapping sets of toxins present in the same venom act synergistically to target the pre- and post-synaptic elements of the neuromuscular junction and maximize the probability of cone snails to successively immobilize and capture the prey. Indeed, the former set of conotoxins is referred to as the 'lightning-strike cabal' and the latter as the 'motor cabal'. These two sets of *Conus* peptides have endowed such slow-moving predator with the talent to capture the agile prey.

The discovery of *Conus* peptides has been greatly accelerated during the past years due to the introduction of sophisticated and effective technical approaches. Many research groups and companies are extraordinarily interested in these small peptides and intend to explore this rich resource [10,11]. An increasing number of *Conus* peptides have been revealed and characterized. Considering different names were often given by different research groups for one peptide, a systematic nomenclature of these active peptides are under urge to ease confusion and bewilderment. Olivera and Cruz have proposed the criterion for systematic classification and unified nomenclature of *Conus* peptides [4]. In brief, the final name of a conotoxin (e.g., κ-conotoxin PVIIA) with a distinct activity consists of a Greek letter to designate its pharmacological action, followed by a hyphen before the word 'conotoxin', a one- or two-letter code representing the *Conus* species, a Roman number indicating its Cys pattern, and terminated with a capital letter for a specific peptide variant. The name of one peptide with unknown pharmacological target (e.g., tx9a) only consists of the species code in lower case letter (s), an Arabic number (designating its Cys pattern) and a small letter for a particular peptide variant. For peptides with the same pharmacological activity but with different cystine framework, a capital letter is introduced to follow the Greek letter to designate a different family (e.g., α and αA, κ and κA). Non-conotoxin families with one or no disulfide bond are usually less diverse and found in fewer species, thus, they are given a name followed by the species code in capital letter (e.g., conopressin-S, contryphan-R and conantokin-G from *C. striatus*, *C. radiatus* and *C. geographus*, respectively). Larger polypeptides (more than 70 aa) are similarly named (e.g., conodipine-M).

The Basis of *Conus* Peptide Diversity

Functional diversity of *Conus* peptides

Conus peptides comprise remarkably diverse classes of

pharmacologically active small peptides. It appears apparent that each *Conus* species has its own distinct repertoire of 50–200 different venom peptides and that the venom peptides from different species are surprisingly divergent in sequence. Thus, more than 50,000 unique peptide sequences lie in over 500 species of predatory cone snails by estimate. Each of these components is believed to have its specific biological target. As mentioned above, the *Conus* venom constitutes a potent pharmacological cocktail, and three distinct targets of *Conus* peptides have been identified till now: ion channels, cell-surface receptors and neurotransmitter transporters. Among them, conotoxins targeting ion channels are under the most extensive studies. Many kinds of ion channels playing pivotal roles in the basic physiological and pathological events have successfully found to be targeted by conotoxins, such as potassium, calcium and sodium channels. Indeed, conotoxins exhibit a variety of activities and could modulate ion channels in various modes, such as block, potentiation or inactivation. κ-, κA- and χ-conotoxins selectively inhibit voltage-gated K^+ channels, while κI-conotoxin BtXA (a systematic name for κ-BtX from *C. betulinus* recently designated in our laboratory) is reported to be the first unique up-modulator of the calcium- and voltage-gated BK channels [12]. Meanwhile, conotoxins preferentially targeting voltage-gated Na^+ channels contain μ-, μO- and δ-families, and the former two inhibit the activity, while the latter one delays the inactivation of these channels, respectively [13]. The variety of tissue selectivity is also observed within a given structural class of peptides (i.e. they share a common disulfide-bonded framework). For example, μ-conotoxin GIIIB binds to skeletal muscle sodium channels with a high affinity and selectivity, whereas μ-conotoxin PIIIA is a preferential inhibitor of neuronal sodium channels [14]. In contrast to the peptides blocking tetrodotoxin-sensitive sodium channels (e.g., μ-conotoxin GIIIB), recently characterized μ-conotoxin SmIIIA [15] and SIIIA (unpublished data) in *C. stercus-muscarum* and *C. striatus* respectively are potent inhibitors of tetrodotoxin-resistant sodium channels. Notably, although ω- and ε-conotoxins possess different cysteine arrangement, they both target N-type voltage-gated Ca^{2+} channels and reduce presynaptic Ca^{2+} influx [16]. Besides, several key cell-surface receptors also encountered their antagonists or agonists in *Conus* venom, that is, conantokins, NMDA receptor antagonists; σ-conotoxins, serotonin receptor antagonists; α- and ψ-conotoxins, competitive and non-competitive antagonists of nicotinic ACh receptors; contulakins, neurotensin-like peptides; conopressins, vasopressin receptor agonists; ρ-conotoxins, antagonists of the G-protein-linked α1-adrenoceptor. Except that, a recently discovered conotoxin family, χ-conotoxins, has been identified to selectively inhibit the activity of neuronal noradrenaline transporter [8]. With the diversity of nicotinic ACh receptor subunits present in the specific prey, individual cone species may produce six or more distinct α-conotoxins and other αA- and ψ-conotoxins, all of which inhibit the postsynaptic nicotinic receptors but in different action modes. Further analysis of α-conotoxin family implies that even in a single *Conus* species there are several distinct members with similar biological function but selectively aiming at a different subtype of a common target [5,17]. Such high target specificity and affinity of *Conus* peptides will offer a broad prospect to discover novel subtypes of the target of interest and to develop potent drugs for the treatment of specific disorders with identified pharmacological targets.

The basic elements for generation of *Conus* peptide diversity

Over tens of millions of years, the venom components of cone snails have undergone rapid and extraordinary evolution, and exhibit an extreme diversify. The versatile activity and unprecedented target selectivity of *Conus* peptides stem from their specific disulfide bond framework combined with hyper-variable intercysteine amino acid sequences. Although the exact molecular mechanisms underlining high interspecific divergence are not well clarified, some facts have been firmly established in recent years. Extensive cDNA analysis of *Conus* peptide precursors elucidates that each *Conus* peptide is encoded by a single mRNA and processed from a precursor usually between 70 and 120 amino acids in length. The prepropeptide precursor has a distinct structural arrangement: a highly conserved signal sequence at the N-terminus (the 'pre-region'), a rather conserved intervening spacer (the 'pro-region') and the hypervariable mature peptide at the C-terminus. Although the mature sequences from different or even the same species can vary greatly with each other, the signal sequences of *Conus* peptides within all members of a same superfamily are extremely conservative, and their pro-regions relatively conserved (Fig. 1).

The conservation of signal peptides and variability of mature *Conus* peptides are intriguing. This juxtaposition of conserved and hypervariable regions within the same translation product reminds us of the mammalian immune system, where antibodies of particular class have defined conserved segments and hypervariable regions. Whether the same mechanism suits to explain the diversity genera-

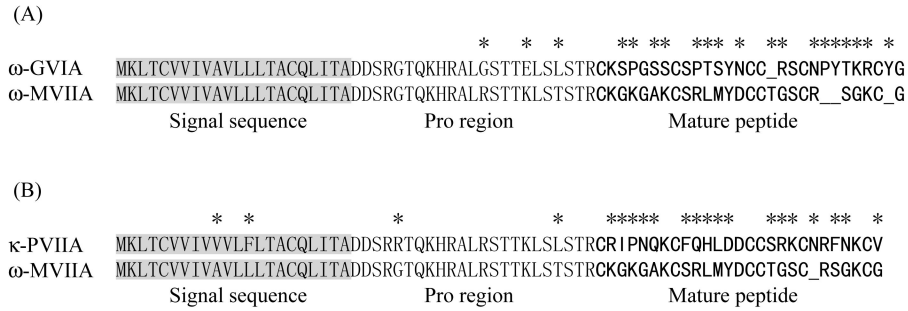

Fig. 1 Comparisons of the precursor sequence of several conotoxins

(A) A comparison of the precursor sequences of two ω-conotoxins from *C. geographus* (ω-GVIA at top) and from *C. magus* (ω-MVIIA at bottom) respectively. (B) A comparison of the precursor sequences of two conotoxins (belonging to different families but the same superfamily) from *C. purpurascens* (κ-PVIIA at top) and *C. magus* (ω-MVIIA at bottom) respectively.

tion of *Conus* peptides, is under investigation. Analysis of *Conus* peptide cDNAs reveals that each mature peptide has its own single copy within the gene, which is not reminiscent of antibody-encoding genes in the mammalian immune system. Up to now, several mechanisms of generating *Conus* peptide diversity are postulated as follows [18–21].

The distinctive gene structure The full-length mRNA of *Conus* peptide is characteristically organized and comprises three segments (encoding signal peptide, pro region and mature peptide, respectively) with untranslational regions at its both termini. The extremely conserved signal sequence is juxtaposed with hyperdivergent mature peptide except the Cys pattern, which has not been found in other systems thus far. Peptide diversification apparently arises by focal hypermutation of the C-terminal toxin-encoding region. Several hypotheses (such as gene recombination, conversion and replication) have been suggested for the molecular basis of focal hypermutation. Recombination between two parental δ-conotoxin genes of *C. magus* successfully generated a novel δ-conotoxin sequence, which brought about the intraspecies diversity [18]. The mature toxin region may adopt many forms of non-synonymous and synonymous replacements, deletions and additions for its hypermutation. What is particularly noteworthy is that all these mutations are restricted within the intercysteine loops whereas the rigid disulfide structural frameworks of the peptides remain unchanged. Replacement of some critical residues within the loops may result in dramatic changes in the target selectivity of these peptides. For instance, a single amino acid substitution ($Ala^{10}Leu$) of α-conotoxin PnIA could sufficiently alter its selectivity of ACh receptor subtypes by two orders of magnitude [22]. The pro-regions of *Conus* peptides, rather conserved, may play important roles in the post-translational modification and the secretion of their mature toxins, whilst the extremely conserved signal sequence may be optimally devised to guide the precursors to specific cell compartment for particular post-translational modification as well as to correctly sort their mature peptides within the cells. In the case of the hydrophobic conotoxin TxVI, its secretion was strongly dependent on its propeptide domain which facilitated the export of this toxin from the endoplasmic reticulum by hitchhiking on sorting receptors [23]. Besides, within the signal region of *Conus* peptide precursor one or two cysteine residues often exist, and these Cys residues was conjectured to have a sequestration role, i.e., to avoid premature or inappropriate disulfide bond formation in the mature toxin region [2,4]. Further characterization of their genome structure may also aid the explanation of the molecular mechanism of *Conus* peptide divergence. Olivera *et al*. [24] documented that the different small exons encoding three segments of *Conus* peptide precursor are separated from each other by relatively large introns. Such charac-teristic exon/intron arrangement makes it possible to yield the accelerated evolution of the C-terminal part and the high conservation of its N-terminal part of the precursor through modulation of gene replication or recombination.

The gene duplication and allelic selection Duba and Palumbi [25,26] sequenced a great number of four-loop conotoxin mRNAs from *C. ebraeus*, *C. abbreviatus* and *C. lividus*. Their results suggested that the gene duplication and allelic selection brought about the diversity of *Conus* peptides possibly linked to ecological diversification and evolutionary success of this genus. Though these three species are all vermivorous and the former two closely related, the mechanisms of their allelic selections are rather

different. Diversifying selection in *C. ebraeus* was detected among alleles at a single locus, whereas the selection in *C. abbreviatus* was apparent among recently diverged loci expressed in single individuals.

The speciation of Conus genus The components found in the venom of one *Conus* species is strikingly different from those present in any other species even very closely related on the basis of other criteria. Each species of cone snail possesses its own distinct and unique array of *Conus* peptides in the venom. Once new species arises, consequently novel *Conus* peptides will come out [24]. An example is that the mature peptides of δ-conotoxins analyzed from different *Conus* species are apparently divergent from each other [18]. Conotoxins are diversifying within species. Presumably, introns separating the different exons encoding the three different regions of *Conus* peptide precursor may contribute to such interspecies diversity of *Conus* peptides to some extent [24].

The variable expression patterns The primary purpose of cone snails to generate toxic peptides is to paralyze prey. When season changes, their prey might vary. Considering the diversity of ion channel and/or receptor subunits present in the different prey types, it is essential for cone snails to produce a wealth of neurotoxins in order to cope with their diverse targets. In accordance with the alteration of prey species at different season, *Conus* may generate and utilize a certain array of *Conus* peptides to effectively and successfully capture prey. Our analysis of the venom components of *C. vexillum* collected from the same place in South China Sea at different seasons demonstrated that both the quantity and type of *Conus* peptides present in the venom varied correspondingly.

The complexity of post-translational modifications It is particularly noteworthy of the remarkable array of post-translational modifications found in *Conus* peptides. In this aspect, Craig *et al.* [20] have presented a comprehensive minireview. The post-translational modifications of *Conus* peptides may be the most sophisticated in the kingdom of animals as some of these modifications are not described outside the *Conus* species. Table 2 listed the post-translational modification forms found in *Conus* peptides to date.

Beyond these extremely complicated modifications, some other uncommon phenomena are observed in *Conus* species. For example, κI-conotoxin BtXA exhibits a pro-region downstream of the C-terminus of its mature toxin instead of the common arrangement of pre-pro-mature peptide [12]. Notably, the post-translational modifications of *Conus* peptides are significant for their biological activities [7,27,28]. For example, the first reported glycopeptide isolated from *Conus* venom, κA-conotoxin SIVA, was characterized from *C. striatus* [27], and the second one, contulakin-G, was from *C. geographus* with a disaccharide attached to its Thr residue at position 10 [28]. *In vivo* studies demonstrated that the biological potencies of these native glycosylated peptides were significantly greater than those of the synthetic nonglycosylated analogues [20, 27,28]. Such rich array of post-translational modifications may provide another overlying level of *Conus* peptide diversity. Presumably, all these modifications require both specific enzymes and their recognition signal sequences (or alternatively characteristic structural features) of cone snails. Although little is known about the molecular mechanisms of most of these post-translational modifications, some unique sequence elements within conopeptide precursors and specialized *Conus* cellular components involved in (or responsible for) these processes have been clearly identified [29–31]. In conantokin-G where five of the six glutamic acid residues are γ-carboxylated, the –1 to –20 sequence of its precursor pro-region acts as a recognition signal and greatly enhances the affinity for the vitamin K-dependent γ-glutamate carboxylase. In another aspect, the pro-region of hydrophobic conotoxin TxVI exhibits a role in the accurate sorting and secretion of this peptide [23].

Table 2 Post-translational modifications of *Conus* peptides

Modification	Enzyme	Example
Hydroxylation of proline	Proline hydroxylase	μ-GIIIA
Amidation of C-terminus	Protein amidating monooxygenase	α-MI
Carboxylation of glutamic acid	γ-glutamate carboxylase	Conantokin-G
Bromination of tryptophan	Bromo peroxidase	Bromocontryphan
Isomerization of tryptophan from *L*- to *D*-form	Tryptophan epimerase	Contryphan
Cyclization of N-terminal Gln	Glutaminyl cyclase	Bromoheptapeptide
Sulfation of tyrosine	Tyrosyl-sulfotransferase	α-EpI
O-Glycosylation	Polypeptide HexNAc transferase	κA-SIVA

Particularly intriguing, Dutton *et al.* [21] recently reported that the synthetic α-conotoxin AuIB with non-native disulfide bond surprisingly had approximately ten times greater potency than the native peptide. Thus, the disulfide bond isomerization may present a new level of *Conus* peptide diversity.

The rapid diversification of *Conus* peptides could be regarded as an optimum evolutionary strategy responsible for the rapid change of their preys, predators and competitors. Cone snails may have acquired their complex venoms over the last 50 million years and generated more and more effective toxins for targeting the key components of the nervous system of their preys. The successive extreme changes in climate and/or geological catastrophe may select for *Conus* peptide hypermutation and may be the key factor in the species richness of the genus *Conus*. With the evolutionary advantage of rapidly optimizing new sets of peptides after geological changes, *Conus* could quickly adapt to a new ecological context. In a historical view, cone snails have utilized a combinatorial library strategy to evolve new peptides in their venoms for survival through successive rounds of strong selection.

Basic Research and Therapeutic Applications

Marine cone snails have successfully evolved to contain a superstore of bioactive small peptides. Over millions of years of evolutionary selection, an incredibly source of these peptides has been developed to target specific ion channels, cell-surface receptors and transporters with extremely high affinities and selectivities. Owing to their high potency and exquisite selectivity, *Conus* peptides are widely used as research tools in the fields of neuroscience and pharmacology, and several conopeptides are at various stages of clinical trials for treatment of a variety of human diseases (Table 3) [10,32]. Since 1996, a large number of patents have been approved for *Conus* peptides and their derivatives.

Targeting ion channels

As discussed above, *Conus* peptides exhibit a rich array of components targeting various kinds of ion channels. The ω-conotoxin family composed of small peptides with 24–31 amino acids in length are constrained to form a rigid conformation cross-linked with three disulfide bonds by incorporating the inhibitor cystine knot (ICK) motif [33]. By virtue of their high affinity and selectivity for different kinds of voltage-gated Ca^{2+} channels (VGCCs), ω-conotoxins are widely employed as probes to discriminate the Ca^{2+} channel subtypes. ω-conotoxin MVIIC and MVIID from *C. magus* could preferentially block the N and P/Q-type VGCCs, whereas ω-conotoxin MVIIA and GVIIA exclusively target the N-type. Meanwhile, Fainzilber *et al.* [34] isolated a novel *Conus* peptide, ω-conotoxin TxVII, which specifically blocked the *L*-type VGCCs. Considering the N-type channels mediate Ca^{2+} influx at nerve terminals and regulate neurotransmitter release, ω-conotoxins blocking specific presynaptic Ca^{2+} channels may have a potential in pain and stroke treatment. For example, ω-conotoxin MVIIA (its synthetic form, Ziconotide), a neuro-specific N-type Ca^{2+} channel blocker with an analgesic and neuroprotective potential, is under development by Elan Pharmaceuticals [35]. In the midst of the blockers of other channels, μ-conotoxins like tetrodotoxin (TTX) play a pivotal role in defining the subtypes of voltage-gated Na^{+} channels. In fact, μ-conotoxin GIIIA exclusively inhibiting Nav1.4 has been applied to resolve the clockwise domain arrangement of sodium channel [36]. Two recently characterized μ-cono-toxins, SmIIIA [15] and SIIIA (unpublished data), which could specifically block TTX-resistant sodium channels, are noteworthy. Since TTX-resistant sodium channels (e.g., Nav1.8 and Nav1.9) are predominantly expressed in sensory neurons and involved in the nociceptive signal transmission, μ-conotoxin SmIIIA and SIIIA may not only provide novel and useful tools to investigate the functional roles of these

Table 3 ***Conus*** **peptides that are on preclinical or clinical trials**

Toxin	Class	Species	Condition	Stage in development	Company website
Vc1.1	α	*C. victoriae*	Neuropathic pain	Preclinical	www.metabolic.com.au
CVID	ω	*C. catus*	Neuropathic pain	Phase II clinical trials	www.amrad.com.au
MVIIA	ω	*C. magus*	Cancer pain	Phase III	www.elan.com
MrIA/B	χ	*C. marmoreus*	Neuropathic pain	Preclinical	www.xenome.com
Contulakin-G	Contulakin	*C. geographus*	Chronic pain	Phase II	www.cognetix.com
Conantokin-G	Conantokin	*C. geographus*	Epilepsy	Preclinical	www.cognetix.com

channels but also have a potential to be developed as efficient pain-killers. Promising in another regard are κ-conotoxins which block potassium channels and might have a wide potential in hypertension, arrhy-thmia or asthma treatment [10]. Nevertheless, a great number of *Conus* peptides with high selectivity to different targets are still remaining to be exploited as pharmacological tools and/or therapeutic agents.

Targeting neurotransmitter receptors and transporters

Cell membrane receptors mediate versatile signal transductions and play crucial roles in physiological and pathological events. By far, cone snails have optimally designed several sets of *Conus* toxins targeting multiple kinds of these receptors. α-conotoxin, the first chemically characterized peptide in *Conus* venom research, is a competitive antagonist of ACh receptor (AChR). This family could not only selectively block nicotinic ACh receptor (nAChR), but also clearly discriminate between muscular and neuronal subclasses of these receptors. In particular, α-conotoxin MII promises to be a useful tool in the study of neuronal β3 subunit-containing AChR [37]. Another α-conotoxin ACV1 (or Vc1.1), recently identified from *C. victoriae*, has attracted considerable attention. In addition to acting as a neuronal-type nAChR antagonist, this novel α-conotoxin can also suppress the vascular responses to unmyelinated sensory nerve C-fiber activation in rats [38]. ACV1 seems to have more potent, non-addictive and long-lasting effects over morphine and does not cause the side effects observed in other pain-killers. Impressively, this peptide can also accelerate tissue repair in the damaged nerves—a unique property not documented yet for any other analgesic agent [39]. Another example of promising antagonist from *Conus* venom is conantokin which is rich in Gla (χ-carboxylglutamic acid) residues and can selectively antagonize the NMDA receptor [40]. These ionotropic glutamate receptors ensure acceleration of Ca^{2+} influx and play critical roles in the pathological events such as CNS trauma, epilepsy, pain and the neuronal cell damage caused by acute brain ischemia. Thus, antagonists of NMDA receptors possess a considerable potential for CNS disorder therapies. For instance, conantokin-G (its synthetic form, CGX-1007), the first identified peptide competitively antagonizing NR2B-containing subtype of NMDA receptors with high selectivity and affinity [41], has exhibited a significant neuroprotective effect in rat models of ischemia and is under development as an antiepileptic agent by Cognetix and Medtronic. At the present time, the NMDA receptor-targeting drugs prescribed in clinic are devoid of selectivity and often evoke unfavorable side effects. As conantokins are capable of selectively targeting different subtypes of NMDA receptors, they could meet specific therapeutic needs with a reduced likelihood of side effects [42]. Also in the case of 5-hydroxytryptamine (5-HT) receptor, no pharmacological probe capable of selectively discrimi-nating among multiple subtypes of this receptor has been found thus far. Fortunately, σ-conotoxin GVIIIA, a novel *Conus* peptide purified from *C. geographus*, can competitively antagonize the 5-HT_3 subtype receptor with high specificity (5-HT_3 receptor is the unique ligand-gated ion channel subtype of the serotonergic receptor family consisting of at least 14 subtypes) [7]. Antagonists of 5-HT_3 receptor have potential antiemetic activity, and may be exploited to minimize chemotherapy-induced nausea and vomiting in clinic. Meanwhile, two new classes of *Conus* peptides specifically blocking α_1-adrenoceptors (ρ-conotoxin TIA) and nor-adrenaline transporters (χ-conotoxin MrIA) were recently characterized from *C. tulipa* and *C. marmoreus*, respectively. Because these peptides exhibit greater selectivity than the current drugs used in clinic, they could also lead to the development of improved therapeutics [8,43].

Pharmacological strategy of cone snails

The venoms of cone snails are a natural resource of peptides with a promising pharmaceutical potential for developing drugs that target ion channels, cell-surface neurotransmitter receptors and transporters. During 50 million years of evolution, cone snails have turned to be expertises in neuropharmacology. Some instructive lessons could be learnt from cone snails for our modern drug design [44].

Small compounds stable and water-soluble Most of *Conus* peptides are small with around 20 residues in length that makes large scale production of their synthetic forms feasible and easy. By comparison, both α-conotoxin ImI and α-bungarotoxin are potent nAChR antagonists, while the former (12 aa) is much smaller than the latter (74 aa). Furthermore, most *Conus* peptides are highly water-soluble and stable, meeting the essential requirement for pharmaceutics.

Stiff framework of the molecule Although *Conus* peptides are mostly small-sized and strikingly hypermutated, their conformations in solution are invariably constrained. Such structural rigidity of these peptides is conferred by the stiff framework constructed upon the highly conserved Cys residues. Even in the case of conantokins which lack disulfide bond, an α-helical conformation is adopted through the intramolecular Gla residues chelated with

calcium ions. Stiff framework will endow drug compounds with stability and selectivity for their 'macro-targets'.

Diverse sequence within a constrained cystine loop Even though most *Conus* peptides within the same superfamily share a common disulfide-bonded framework, they exhibit a variety of different pharmacological specificities. Hypermutations occurring within the intercystine loops result in the diverse loop sequences (and/or sizes), and bestow high selectivity on these peptides to discriminate the different subtypes of common 'macro-targets'. For example, ω-conotoxin GVIA and MVIIB could distinguish among various subtypes of voltage-gated calcium channels with a selectivity ratio of $1:10^8$ (N-type over non-N-type subtypes) [45]. It was hypothesized that the common constrained framework endowed these peptides with the activity to target the same 'macrosite', while the hypermutated sequences within the intercystine loops might contribute to their extreme preference and binding affinity for different 'microsite' [46]. In fact, focal modifications of lead compounds can generate drugs with a high selectivity and less side effects.

Synergy of operations Cone snails take advantage of the synergetic effects of different *Conus* peptides aiming at various targets to capture their preys efficiently. Two distinct phases of their prey capture are elicited by different sets of *Conus* peptides (e.g., 'lightning-strike cabal' and 'motor cabal'). Such strategy of synergy has been adopted in the treatment of diseases (i.e. different kinds of drugs with different pharmacological efficiencies are often under combined administration in clinic).

Future Prospects

Conus peptides comprise a vast array of active peptides with over 50,000 estimated components. Given that only 0.2% of this library was published in the scientific literature, most of this 'arsenal' remain to be explored. In the retrospect of *Conus* venom research, Craig Clark of the University of Utah inspiringly injected venom fractions directly into the central nervous system of mammals, instead of using the standard intraperitoneal injection. Intracranial injection of *Conus* venom fractions in mice elicited the amazing array of different behavioral phenotypes revealing the true pharmacological diversity of *Conus* venoms [4,47]. With this recording of many diverse symptoms elicited in mice, several *Conus* peptides were successfully characterized from *C. geographus*, such as a 'shaker' peptide (ω-conotoxin GVIA), 'scratcher' peptide (conopressin-G), 'sleeper'peptide (conantokin-G) and 'sluggish' peptide (contulakin-G). Today, with rapid development of effective methodology and sensitive bioassay, many novel *Conus* active peptides have been characterized not only by chemical but also by molecular biological approaches. Indeed, a great number of novel *Conus* peptides have been discovered over these years by means of cDNA cloning [38]. Meanwhile, the extensive structure-function studies of these diverse peptides with various pharmacological potential and target selectivity are underway, and will provide a substantial basis to understand the mechanisms of the 'ligand-receptor' interactions and for the development of new probes and therapeutic agents [9,48]. With the novel ion channels and cell-surface receptors/transporters (especially G-protein coupled receptors) rapidly discovered, their putative 'exogenous ligands' present in the venoms of cone snails will accelerate the understanding of the fundamental roles of these elements in physiology and disease. Furthermore, a given structural framework of *Conus* peptides already proved to target certain subtype of receptor or ion channel might serve as an excellent template for the development of novel therapeutics with higher selectivity and less side effects in pharmaceutical industry.

Several postulates have been presented to explain the generation of conotoxin diversity [18–21]. In future, extensive analysis of the cone snail genes (sequence and structure characteristics of their mRNAs and genome) may provide a fundamental basis to elucidate the mechanisms of *Conus* peptide biosynthesis and diversity. Their complicated post-translational modifications, one extremely sophisticated issue, also remain to be clarified. Though some progresses have been made in these aspects, massive efforts should be laid on the biological exploration of these rich pharmaceutical resources.

References

1 le Gall F, Favreau P, Richard G, Letourneux Y, Molgo J. The strategy used by some piscivorous cone snails to capture their prey: The effects of their venoms on vertebrates and on isolated neuromuscular preparations. Toxicon, 1999, 37(7): 985–998
2 Olivera BM. E.E. Just Lecture, 1996. *Conus* venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology. Mol Biol Cell, 1997, 8(11): 2101–2109
3 Terlau H, Shon KJ, Grilley M, Stocker M, Stuhmer W, Olivera BM. Strategy for rapid immobilization of prey by a fish-hunting marine snail. Nature, 1996, 381(6578): 148–151
4 Olivera BM, Cruz LJ. Conotoxins, in retrospect. Toxicon, 2001, 39(1): 7–14
5 McIntosh JM, Jones RM. Cone venom—from accidental stings to deliberate injection. Toxicon, 2001, 39(1): 1447–1451
6 Jimenez EC, Shetty RP, Lirazan M, Rivier J, Walker C, Abogadie FC,

Yoshikami D *et al*. Novel excitatory *Conus* peptides define a new conotoxin superfamily. J Neurochem, 2003, 85(3): 610–621
7 England LJ, Imperial J, Jacobsen R, Craig AG, Gulyas J, Akhtar M, Rivier J *et al*. Inactivation of a serotonin-gated ion channel by a polypeptide toxin from marine snails. Science, 1998, 281(5376): 575–578
8 Sharpe IA, Gehrmann J, Loughnan ML, Thomas L, Adams DA, Atkins A, Palant E *et al*. Two new classes of conopeptides inhibit the α1-adrenoceptor and noradrenaline transporter. Nat Neurosci, 2001, 4(9): 902–907
9 Jones RM, Bulaj G. Conotoxins—new vistas for peptide therapeutics. Curr Pharm Des, 2000, 6(12): 1249–1285
10 Shen GS, Layer RT, McCabe RT. Conopeptides: From deadly venoms to novel therapeutics. Drug Discov Today, 2000, 5(3): 98–106
11 Heading CE. *Conus* peptides and neuroprotection. Curr Opin Investig Drugs, 2002, 3(6): 915–920
12 Fan CX, Chen XK, Zhang C, Wang LX, Duan KL, He LL, Cao Y *et al*. A novel conotoxin from *Conus betulinus*, κ-BtX, unique in cysteine pattern and in function as a specific BK channel modulator. J Biol Chem, 2003, 278(15): 12624–12633
13 McIntosh JM, Hasson A, Spira ME, Gray WR, Li W, Marsh M, Hillyard DR *et al*. A new family of conotoxins that blocks voltage-gated sodium channels. J Biol Chem, 1995, 270(28): 16796–16802
14 Safo P, Rosenbaum T, Shcherbatko A, Choi DY, Han E, Toledo-Aral JJ, Olivera BM *et al*. Distinction among neuronal subtypes of voltage-activated sodium channels by μ-conotoxin PIIIA. J Neurosci, 2000, 20(1): 76–80
15 West PJ, Bulaj G, Garrett JE, Olivera BM, Yoshikami D. μ-conotoxin SmIIIA, a potent inhibitor of tetrodotoxin-resistant sodium channels in amphibian sympathetic and sensory neurons. Biochemistry, 2002, 41(51): 15388–15393
16 Rigby AC, Lucas-Meunier E, Kalume DE, Czerwiec E, Hambe B, Dahlqvist I, Fossier P *et al*. A conotoxin from *Conus* textile with unusual posttranslational modifications reduces presynaptic Ca^{2+} influx. Proc Natl Acad Sci USA, 1999, 96(10): 5758–5763
17 McIntosh JM, Santos AD, Olivera BM. *Conus* peptides targeted to specific nicotinic acetylcholine receptor subtypes. Annu Rev Biochem, 1999, 68: 59–88
18 Espiritu DJ, Watkins M, Dia-Monje V, Cartier GE, Cruz LJ, Olivera BM. Venomous cone snails: Molecular phylogeny and the generation of toxin diversity. Toxicon, 2001, 39(12): 1899–1916
19 Olivera BM, Rivier J, Clark C, Ramilo CA, Corpuz GP, Abogadie FC, Mena EE, Woodward SR, Hillyard DR, Cruz LJ. Diversity of *Conus* neuropeptides. Science, 1990, 249(4966): 257–263
20 Craig AG, Bandyopadhyay P, Olivera BM. Post-translationally modified neuropeptides from Conus venoms. Eur J Biochem, 1999, 264(2): 271–275
21 Dutton JL, Bansal PS, Hogg RC, Adams DJ, Alewood PF, Craik DJ. A new level of conotoxin diversity, a non-native disulfide bond connectivity in α-conotoxin AuIB reduces structural definition but increases biological activity. J Biol Chem, 2002, 277(50): 48849–48857
22 Luo S, Nguyen TA, Cartier GE, Olivera BM, Yoshikami D, McIntosh JM. Single-residue alteration in α-conotoxin PnIA switches its nAChR subtype selectivity. Biochemistry, 1999, 38(44): 14542–14548
23 Conticello SG, Kowalsman ND, Jacobsen C, Yudkovsky G, Sato K, Elazar Z, Petersen CM *et al*. The prodomain of a secreted hydrophobic mini-protein facilitates its export from the endoplasmic reticulum by hitchhiking on sorting receptors. J Biol Chem, 2003, 278(29): 26311–26314
24 Olivera BM, Walker C, Cartier GE, Hooper D, Santos AD, Schoenfeld R, Shetty R *et al*. Speciation of cone snails and interspecific hyperdivergence of their venom peptides. Potential evolutionary significance of introns. Ann NY Acad Sci, 1999, 870: 223–237
25 Duda TF Jr, Palumbi SR. Molecular genetics of ecological diversification: Duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. Proc Natl Acad Sci USA, 1999, 96(12): 6820–6823
26 Duda TF Jr, Palumbi SR. Evolutionary diversification of multigene families: Allelic selection of toxins in predatory cone snails. Mol Biol Evol, 2000, 17(9): 1286–1293
27 Craig AG, Zafaralla G, Cruz LJ, Santos AD, Hillyard DR, Dykert J, Rivier JE *et al*. An O-glycosylated neuroexcitatory *Conus* peptide. Biochemistry, 1998, 37(46): 16019–16025
28 Craig AG, Norberg T, Griffin D, Hoeger C, Akhtar M, Schmidt K, Low W *et al*. Contulakin-G, an O-glycosylated invertebrate neurotensin. J Biol Chem, 1999, 274(20): 13752–13759
29 Bandyopadhyay PK, Colledge CJ, Walker CS, Zhou LM, Hillyard DR, Olivera BM. Conantokin-G precursor and its role in γ-carboxylation by a vitamin K-dependent carboxylase from a *Conus* snail. J Biol Chem, 1998, 273(10): 5447–5450
30 Begley GS, Furie BC, Czerwiec E, Taylor KL, Furie GL, Bronstein L, Stenflo J *et al*. A conserved motif within the vitamin K-dependent carboxylase gene is widely distributed across animal phyla. J Biol Chem, 2000, 275(46): 36245–36249
31 Czerwiec E, Begley GS, Bronstein M, Stenflo J, Taylor K, Furie BC, Furie B. Expression and characterization of recombinant vitamin K-dependent γ-glutamyl carboxylase from an invertebrate, *Conus* textile. Eur J Biochem, 2002, 269(24): 6162–6172
32 Nelson L. Venomous snails: One slip, and you're dead. Nature, 2004, 429(6994): 798–799
33 Norton RS, Pallaghy PK. The cystine knot structure of ion channel toxins and related polypeptides. Toxicon, 1998, 36(11): 1573–1583
34 Fainzilber M, Lodder JC, van der Schors RC, Li KW, Yu Z, Burlingame AL, Geraerts WP *et al*. A novel hydrophobic ω-conotoxin blocks molluscan dihydropyridine-sensitive calcium channels. Biochemistry, 1996, 35(26): 8748–8752
35 Jain KK. An evaluation of intrathecal ziconotide for the treatment of chronic pain. Expert Opin Investig Drugs, 2000, 9(10): 2403–2410
36 Li RA, Ennis IL, French RJ, Dudley SC Jr, Tomaselli GF, Marban E. Clockwise domain arrangement of the sodium channel revealed by μ-conotoxin GIIIA docking orientation. J Biol Chem, 2001, 276(14): 11072–11077
37 McIntosh JM, Gardner S, Luo S, Garrett JE, Yoshikami D. *Conus* peptides: Novel probes for nicotinic acetylcholine receptor structure and function. Eur J Pharmacol, 2000, 393(1-3): 205–208
38 Sandall DW, Satkunanathan N, Keays DA, Polidano MA, Liping X, Pham V, Down JG *et al*. A novel α-conotoxin identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves *in vivo*. Biochemistry, 2003, 42(22): 6904–6911
39 Brown M. Killer snails ease the pain. Drug Discov Today, 2002, 7(17): 885–886
40 Castellino FJ, Prorok M. Conantokins: inhibitors of ion flow through the *N*-methyl-*D*-aspartate receptor channels. Curr Drug Targets, 2000, 1(3): 219–235
41 Donevan SD, McCabe RT. Conantokin G is an NR2B-selective competitive antagonist of *N*-methyl-*D*-aspartate receptors. Mol Pharmacol, 2000, 58(3): 614–623
42 Malmberg AB, Gilbert H, McCabe RT, Basbaum AI. Powerful antinociceptive effects of the cone snail venom-derived subtype-selective NMDA receptor antagonists conantokins G and T. Pain, 2003, 101(1-2): 109–116
43 Harvey AL. Toxins 'R' Us: more pharmacological tools from nature's superstore. Trends Pharmacol Sci, 2002, 23(5): 201–203
44 Olivera BM. Hillyard DR, Marsh M, Yoshikami D. Combinatorial peptide libraries in drug design: Lessons from venomous cone snails. Trends Biotechnol, 1995, 13(10): 422–426
45 Olivera BM, Miljanich GP, Ramachandran J, Adams ME. Calcium channel diversity and neurotransmitter release: The ω-conotoxins and ω-agatoxins.

Annu Rev Biochem, 1994, 63: 823–867
46 Olivera BM, Rivier J, Scott JK, Hillyard DR, Cruz LJ. Conotoxins. J Biol Chem, 1991, 266(33): 22067–22070
47 Olivera BM, Cruz LJ, Yoshikami D. Effects of *Conus* peptides on the behavior of mice. Curr Opin Neurobiol, 1999, 9(6): 772–777
48 Harvey A. From demons to darlings: Drugs from venoms. Drug Discov Today, 1998, 3(12): 531–532

Edited by
Zu-Xun GONG