Minireview

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Conotoxins*

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Many successful animal and plant families have developed distinctive biochemical strategies; one of the more unusual examples is found in a group of marine gastropods, the cone snails (*Conus*) (1). These animals have evolved a specialized biochemistry of small constrained peptides, the conotoxins. These peptides are the direct translation products of genes (2). However, because they are small enough for direct chemical synthesis and sufficiently constrained for three-dimensional conformation determination, conotoxins bridge protein chemistry and molecular genetics. Furthermore, the strategy that the cone snails have evolved over millions of years for the generation and design of an enormous array of small peptide ligands, each with high affinity and specificity for a particular receptor protein target, may be adaptable for use *in vitro*.

Natural History of Cone Snails

The focus of this minireview is the small peptides made in the venoms of the cone snails (*Conus*). On a geological time scale, the true cones are a recently evolved group. The oldest verifiable *Conus* fossils occur well after the Cretaceous extinction (3), an event resulting in the disappearance of dinosaurs on land and the ammonites in marine environments. Just as the extinction of dinosaurs provided an opportunity for the rise of the mammals, the extinction of the ammonites was probably a key factor for the success of *Conus*. Ammonites were believed to be among dominant predators in rich, shallow water marine communities, an ecological niche occupied by the cone snails today. The genus *Conus* has been expanding at an impressive rate; the ~500 living species make it perhaps the largest single molluscan genus (see Fig. 1).

Although individual *Conus* species can be highly specialized, as a whole the genus shows a remarkably broad phylogenetic range of prey. At least five different phyla of animals are envenomated by cone snails; there are large numbers of *Conus* species which feed only on polychaete worms, other snails, or fish (4). Slow moving snails might not be expected to capture fish successfully, but dozens of *Conus* species eat nothing else. Observing a fish-hunting cone such as *Conus striatus* capture prey is a memorable sight. In the presence of fish, the snail extends its long threadlike proboscis which serves as a fishing line. A hollow, arrow-shaped tooth is ejected at the tip of the proboscis and is used to harpoon the fish (see Fig. 2) and inject the venom. The fish typically jerks suddenly after being struck but remains tethered through the proboscis. A good strike causes the fish to be immobilized within 1 or 2 s, unable to use its major fins. Total paralysis is effected a few seconds later, but often the fish has been engulfed by the snail into its distensible stomach even before this has occurred. The potent venom is made in a long duct and expelled using a muscular bulb. Although the ~500 *Conus* species hunt different prey and have different foraging strategies, all inject venom through a harpoon-like tooth to immobilize prey. One species, *Conus geographus*, is so venomous that two-thirds of human stinging cases are fatal.

Overview of Conus Peptides

The biologically active agents in *Conus* venoms are unusually small peptides, 10–30 amino acids in length. Most peptides are multiply disulfide-bonded; small loops of 1–6 amino acids are interspersed between the disulfide-bonded Cys residues. There is a large array of different peptides in every venom, and each appears to be specifically targeted to a particular receptor. The profile shown in Fig. 3 is typically obtained upon analysis of a *Conus* venom fraction; a wide range of biological activities is observed. Physiological targets have been identified for several peptides found in *Conus* venoms (see Table I and Refs. 1 and 5). However, for most peptides in *Conus* venoms already biochemically characterized (over 70 peptides so far, from 10 venoms), the receptor targets remain unknown. The full complexity of any single *Conus* venom has not yet been determined; there may well be over 100 different peptides in the more complex venoms.

Each *Conus* species has a venom with a distinct pharmacological profile. For example, a major component of *C. geographus* venom is conantokin-G, which causes sleep in young mice and hyperactivity in older mice and targets to the NMDA[1] receptor (6, 7). The venom of another fish hunter, *C. striatus* does not exhibit this activity. Conversely, *C. striatus* venom has a major excitotoxin not present in *C. geographus* venom. Although both species make peptides targeted to the acetylcholine receptor and to voltage-sensitive calcium channels, each venom has a large subset of pharmacologically distinct entities. Additional pharmacologically active factors from different *Conus* venoms have been described including agents with α-adrenergic (8) and cholinomimetic (9) effects, as well as purified components with potent effects on smooth and cardiac muscle systems (10–13). No detailed sequence information has yet been published for these, but they appear to be distinct from the conotoxin classes in Table I.

Despite the great diversity of peptides in *Conus* venoms, one striking structural feature is the pattern of Cys residues. A large fraction of *Conus* peptides exhibits one of three characteristic arrangements of cysteine residues: the "standard" 2-loop, 3-loop, and 4-loop conotoxin frameworks (see Table II). The major 4-loop framework (C– – –C– – –CC– – –C– – –C) has been identified in over 20 *Conus* peptides with a wide range of pharmacological effects. Alternative arrangements, some characteristic of Cys-rich peptides in other systems, are not found. For example, an alternative 4-loop framework found in mammalian defensins (14) (C– – –C– – –C– – –C– – –CC) is not present in any *Conus* peptide.

There are a number of *Conus* peptides that lack disulfide bonding altogether. These may assume specific conformations through mechanisms other than multiple disulfide linkages. An example are the conantokins, which target to NMDA receptors. In these peptides γ-carboxyglutamate residues are believed to induce an α-helical conformation in the presence of calcium ions (15). Thus, although the great majority of venom peptides have multiple disulfide bonds, there may be alternative strategies for stabilizing high affinity binding conformations in a minor fraction of *Conus* peptides.

Hypervariability of Conotoxin Homologs

Analysis of cDNA clones of conotoxins has led to the conclusion that a specialized genetic mechanism has evolved in *Conus*

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[1] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; HPLC, high performance liquid chromatography.

FIG. 1. **Peptide specialists: some of the ~500 different cone snail species.** Each *Conus* species produces a venom with its own characteristic set of diverse small constrained peptides. Although most venoms have not yet been biochemically characterized, each should yield distinctive peptide ligands which specifically bind cell-surface receptors or ion channels. There is remarkable hypervariability between peptide sequences from venoms of different cone species. Possibly, a similar hypervariability generating mechanism serves to produce the strikingly diverse shell patterns as well. Photograph by Kerry Matz.

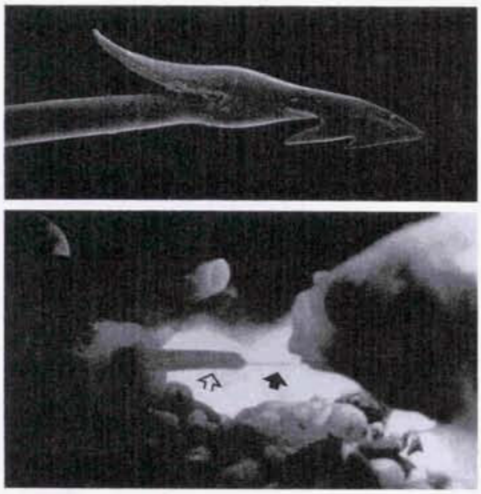

FIG. 2. *Top panel*, the tip of the harpoon-like tooth of *Conus obscurus*. The barbed, hollow tooth is used for injecting venom into the fish prey. Scanning electron micrograph by Dr. Ed King and Chris Hopkins. *Lower panel*, a specimen of *C. striatus* has harpooned a fish which is immobilized and is being drawn toward the mouth of the snail. The *filled arrow* indicates the harpoon tooth through which venom was injected; the *empty arrow* shows the proboscis which has been largely pulled back into the mouth of the snail. The structure at the *top* of the photograph is the siphon, used by these largely nocturnal snails use to locate prey. Photograph by Kerry Matz.

to generate hypervariability in the loop regions between Cys residues of the standard frameworks (see Fig. 4) (1, 2). This may explain why conotoxins have highly conserved arrangements of cysteine residues; *Conus* peptides with new pharmacologic specificity and biological roles are most likely to evolve with one of the standard conotoxin frameworks because of the hypervariability-generating mechanism. In the *Conus* peptide system, extreme sequence hypervariability is observed between functionally homologous conotoxins. The same mechanism that gives rise to the wide variety of pharmacologically different conotoxins may also be responsible for sequence hypervariability within each pharmacological class. A set of peptide ligands from a single genus, targeted to the same binding pocket of a particular receptor, would normally be expected to be highly conserved in primary sequence; instead, a remarkable divergence is found.

Homologous peptides from two venoms that both target to presynaptic Ca^{2+} channels are shown in Table IIIA (16). If the sequences of these peptides, ω-conotoxins GVIA and MVIIA, are aligned, less than one-third of the non-cysteine amino acids are identical. Furthermore, the amino acids in corresponding loops are strikingly different; for example, ω-conotoxin GVIA has 3 residues of hydroxyproline, whereas ω-conotoxin MVIIA has none. Despite these substantial sequence differences, both ω-conotoxins target the same subset of calcium channels and elicit identical biological effects in most phylogenetic systems (17). Presumably, these peptides were both evolved to cause paralysis by inhibiting presynaptic calcium channels at fish neuromuscular junctions, since both come from fish-hunting *Conus*.

It is noteworthy that most of the amino acids conserved between ω-conotoxins GVIA and MVIIA are also conserved in a peptide with entirely different pharmacological specificity, the King-Kong peptide from *Conus textile* (2, 18). If all ω-conotoxin sequences are aligned, only the 6 cysteine residues and one glycine moiety are conserved (see Table III); these are all present in the King-Kong peptide. Thus, the high binding specificity of ω-conotoxins for calcium channels at vertebrate presynaptic termini must be due to the variable loop sequences and not the amino acid residues conserved in both ω-conotoxins and the King-Kong peptide (such as the Cys framework).

Considerable polymorphism occurs even in the same venom. Two α-conotoxins from *C. striatus* (19, 20) are shown in Table IIIB. The Cys residues are conserved, but fully two-thirds of the other amino acids differ in the two peptides. The hypervariability of conotoxin homologs extends to peptides which are not disulfide-bonded. Thus, as shown in Table IIIC, conantokins from two different *Conus* species, which are essentially identical in biological activity, are highly divergent in primary sequence (21).

TABLE I

Identified receptor targets of conotoxins

The detailed sequences of all peptides are shown in Ref. 16.

Targets identified	Conotoxin example from *C. geographus*	Size of conotoxin[a]	No. of disulfide bonds
Voltage-sensitive Ca^{2+} channel	ω-Conotoxin GVIA	27	3
Acetylcholine receptor	α-Conotoxin GI	13	2
Voltage-sensitive Na^{+} channel	μ-Conotoxin GIII	22	3
Vasopressin receptor	Conopressin-G	9	1
NMDA receptor	Conantokin-G	17	0

[a] No. of amino acids.

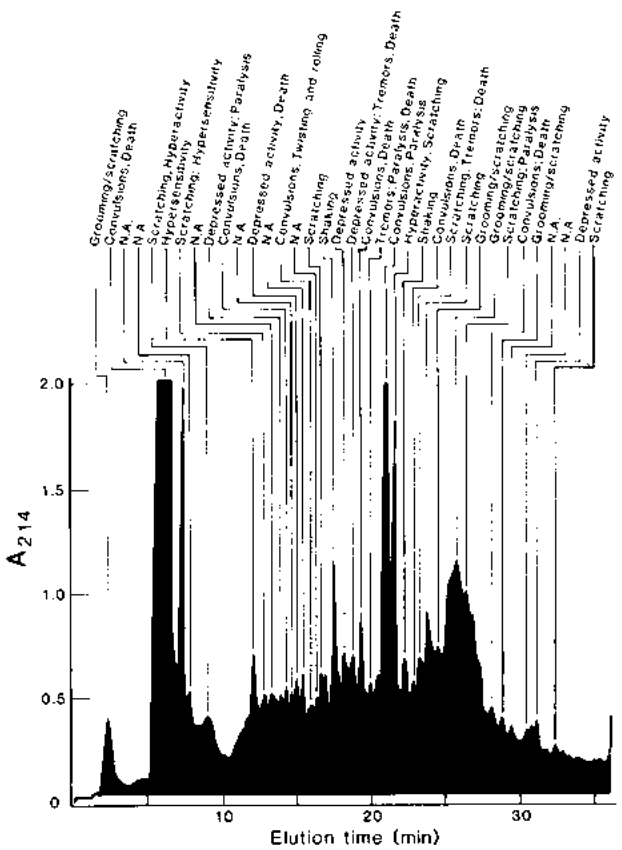

FIG. 3. **An HPLC analysis of a peptide fraction from *Conus magus* venom.** A peptide fraction from crude *C. magus* venom was obtained after size fractionation on Sephadex G-25 and reverse phase HPLC carried out as previously described. Each peak was assayed by intracranial injection of 0.5–2 nmol into mice (assuming average absorbance and molecular weight). Symptoms obtained are indicated above each peak. The two peaks that induce shaking are ω-conotoxins; *N.A.* indicates no biological activity observed.

TABLE II

Major conotoxin frameworks

Framework	Examples[a]
"4-loop" framework: 1 2 3 4 C– – –C– – –CC– – –C– – –C	ω-Conotoxins (*C. geographus*, *C. magus*); "King-Kong" peptides (*C. textile*)
"3-loop" framework: 1 2 3 CC– – –C– – –C– – –CC	μ-Conotoxins (*C. geographus*); "scratcher" peptide (*C. textile*)
"2-loop" framework: 1 2 CC– – –C– – –C	α-Conotoxins (*C. geographus*, *C. striatus*)

[a] Detailed sequences of all examples are in Ref. 1.

Conotoxin Sequence Degeneracy and Receptor-Ligand Interactions

Why is it possible for peptides with strikingly different primary sequences (such as ω-conotoxins GVIA and MVIIA) to target the same binding sites? Except for the conserved disulfide frameworks, which are demonstrably not the primary determinants of binding specificity, conotoxin homologs are surprisingly diverse in primary sequence. One explanation is that the conotoxin surfaces that interact with the receptor target (the "pharmacophore" in the language of pharmaceutical chemistry) have the same conformation despite divergent primary sequences. In this view, there are degenerate ways to get congruent conformations, and amino acid identity in specific positions is not obligatory.

Alternatively, ligands the size of conotoxins may interact with a "macrosite" on the receptor target that contains a number of "microsites." Each microsite could contribute to binding affinity upon contact with the ligand. The essence of this hypothesis is that only a fraction of all potential microsites actually make focal

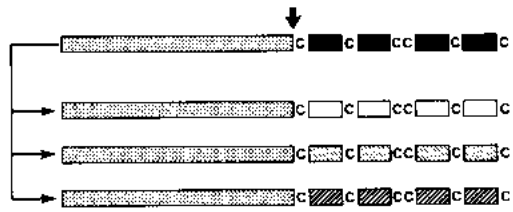

FIG. 4. **Evolution of new conotoxins.** cDNA cloning has indicated that although the N-terminal end of the conotoxin precursors is highly conserved, the cone snails have a genetic mechanism for introducing rapid sequence changes specifically in loops (represented as *black bars* in the original peptide) between cysteine residues. The *arrow* represents the site of proteolytic cleavage to release the mature Cys-rich conotoxin from a prepropeptide precursor. By switching loops between cysteine residues at the gene level (perhaps by a cassette switching mechanism), three new peptides could be generated. The conservation of both the excised N-terminal preproregion and the Cys residues in the mature toxin probably guarantees that specific disulfide bonding is conserved. However, the new peptides may either have the same pharmacological specificity or entirely different pharmacological specificity from the original peptide and from each other.

TABLE III

Hypervariability of conotoxins

Sequences given are from Refs. 1, 17, and 19–21.

A. ω-Conotoxins from *C. geographus* and *C. magus*	
ω-Conotoxin GVIA	CKSPGSSCSPTSYNCCRS-CNPYTKRCY**
ω-Conotoxin MVIIA	CKGKGAKCSRLMYDCCTGSCRSGK--C*
AA identities in 12 ω-conotoxins sequenced	C G C CC C C
King-Kong peptide (not an ω-conotoxin)	WCKQSGEMCNLLDQNCCDGYCIVLV--CT
B. α-conotoxins from *C. striatus*	
α-Conotoxin SI	ICCNPACGPKYSC*
α-Conotoxin SIA	YCCHPACGKNFDC*
C. Conantokins from *C. geographus* and *C. tulipa*	
Conantokin-G	GEγγLQγNQγLIRγKSN*
Conantokin-T	GEγγYQKMLγNLRγAEVKKNA*

**, C-terminal amidation; P, hydroxyproline; γ, γ-carboxyglutamate.

contact with determinants on the conotoxin. Thus, two different conotoxins with the same pharmacological specificity could contact a different subset of microsites within the same macrosite. Therefore, a large number of diverse peptide structures could potentially bind a macrosite. An important prediction of this hypothesis is that pharmacologically homologous conotoxins with divergent primary sequences would not be conformationally identical, even at the contact surface with the receptor (Fig. 5).

Conotoxins have great utility for studying cell-surface receptors, particularly in the nervous sytem. In general, the receptor system under study is not the natural physiological target but one that is evolutionarily related. For example, ω-conotoxin GVIA is widely used to study mammalian central nervous system calcium channels, but not fish calcium channels, the natural target. In the macrosite model above, a receptor in the same class as the natural target could have many microsites conserved but a subset that may have diverged. Only the subset of conotoxin homologs with direct focal contact would have altered receptor affinity when a particular microsite is altered. Thus, a set of conotoxin homologs should all bind the natural target with high affinity; if tested on an evolutionarily related receptor, the set would not behave uniformly but in an eclectic fashion, some with high affinity and some not binding at all. There are experimental observations consistent with this prediction, *i.e.* the dramatically different behavior of α-conotoxins GI and SI on mammalian neuromuscular synapses, and of ω-conotoxin GVIA and MVIIA on amphibian neurotransmission. Such results lend some credence to the macrosite model in Fig. 5. However, the two models above are not mutually exclusive; both pharmacophore conformation degeneracy on the ligand and alternative microsite contacts on the receptor could conceivably contribute to the sequence divergence observed between any two conotoxin homologs.

Perspectives and Future Directions

At the present time, only a few *Conus* venoms have been surveyed, and even in the best characterized venom, only a minor

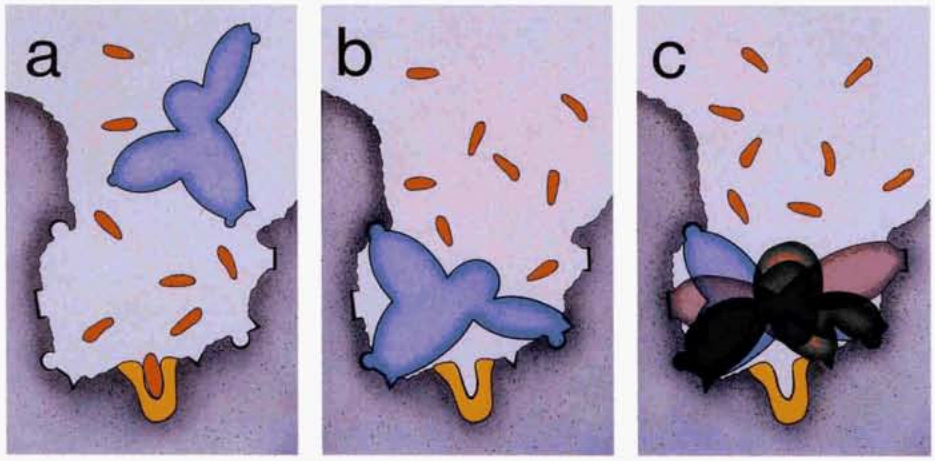

FIG 5. **The *Conus* toxin macrosite model.** A representation of a receptor binding pocket with a number of microsites which can potentially make focal contacts with conotoxin ligands is shown. The diagram illustrates a receptor with an endogenous ligand agonist (an example is the acetylcholine receptor); the endogenous ligands are the *orange* blobs, and the *yellow* region is the agonist binding site. The *middle panel* illustrates a conotoxin-blocking endogenous ligand binding, making three microsite focal contacts. As shown in the *right panel*, the macrosite can be alternatively occupied by conotoxins with different primary sequences, each making a different subset of focal contacts. In this way, even peptides with highly divergent sequences compete for binding to the same receptor pocket. In the example shown *blue* and *green* conotoxins share two focal contacts, while each shares one with the *purple* conotoxin. All three would serve as antagonists of this receptor.

fraction of peptides has been biochemically or pharmacologically characterized. However, this data base still permits a number of generalizations. First, there is remarkable pharmacological and biochemical diversity of small constrained peptides in each *Conus* venom. In *Conus geographus* venom, small peptide ligands target calcium channels, sodium channels, acetylcholine receptors, and NMDA receptors. Even more intriguing are the much larger number of biologically active peptides in the same venom for which receptors have not yet been identified.

In addition to the peptide diversity in an individual venom, an amazing sequence hypervariability between venoms is observed. No two *Conus* species have yet been found with the same conotoxin sequence. The present data base is best for the paralytic conotoxins; it seems reasonable to expect that every *Conus* venom will contain conotoxins directly paralytic to the prey. One obvious class of paralytics is conotoxins which inhibit acetylcholine receptors at neuromuscular junctions. Such agents have been described in all fish-hunting species examined and are very likely found in worm-hunting and mollusc-hunting *Conus* venoms as well. However, the toxins in fish-hunting species are presumably selected to inhibit fish acetylcholine receptors, while the corresponding toxins in the venoms of vermivorous *Conus* species would interact optimally with worm receptors. We can extrapolate from the data already collected that an acetylcholine receptor-targeted conotoxin in one species will have a significantly different sequence from that in any another *Conus* species. Thus, for the genus as a whole, there should be literally over a thousand different small peptides targeted to acetylcholine receptors. It seems likely that large sets of *Conus* peptides will be similarly targeted to many other receptors and ion channels.

The pharmacological potential of such substantial collections of small constrained peptides targeting to one class of receptors is immense. The acetylcholine receptor-targeted peptides can be tested on various acetylcholine receptors in different phylogenetic systems, such as the set of neuronal receptors in mammalian brain. While we cannot predict which peptides in the collection will have high affinity for a particular mammalian central nervous system acetylcholine receptor subtype, the natural repertoire of conotoxins should provide a rich source of ligands for discriminating between different receptor target subtypes.

As more information is collected about conotoxin design and synthesis in the natural system, it becomes increasingly feasible to apply similar strategies to generate conotoxin-like molecules *in vitro*. One particularly promising approach is to combine conotoxin biochemistry with newly developed peptide screening methods, such as the fUSE phage-peptide library technique of Scott and Smith (22). Recently, it was shown that the addition of a conotoxin module as a fusion to a phage coat protein did not affect phage viability (23). Thus, it is feasible to clone billions of conotoxin-like sequences onto vectors like fUSE phage and screen the library of conotoxin-like modules for interaction with cloned receptor targets. The phage that do exhibit affinity for the receptor target can then be amplified, the DNA sequenced, and the predicted disulfide-rich conotoxin-like peptide synthesized and tested for binding to the receptor target. Modification experiments to determine the focal contact points with the receptor are also feasible. Screens for conotoxins with any pharmacological specificity desired can be easily designed, *i.e.* small peptides that have very high affinity for one receptor subtype but which do not bind other closely related subtypes at all. Since these peptides will be constrained conformationally because of multiple disulfide bonds, their three-dimensional conformation could then be analyzed. This opens the door to pure chemical applications; for example, appropriate peptidomimetic derivatives can then be designed. The combination of a molecular genetic approach to allow screening of billions of sequences with the insight provided by the conotoxin system bridges molecular genetics and chemistry in a way that should permit exciting future applications in the pharmaceutical industry and many other areas of biotechnology.

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