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Conus Peptides: Biodiversity-based Discovery and Exogenomics*

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The venoms of the ~700 species of predatory cone snails (genus *Conus*) are being systematically characterized. Each *Conus* species contains 100–200 small, highly structured venom peptides (colloquially known as conotoxins), which are synthesized and secreted in a venom duct (for overviews, see Refs. 1–3). The biomedical potential of these small venom peptides is now well established; recent developments are summarized below. Additionally, the genetic basis and biological rationale for *Conus* peptide diversity is addressed.

Conus peptide genes belong to a general class whose gene products are targeted to other organisms; the extensive molecular analysis carried out on the *Conus* genes revealed that these diversify extremely rapidly. Understanding this class of genes may require a specialized framework with features distinct from conventional genomics, which we will refer to as "exogenomics" (the gene products are targeted exogenously rather than endogenously). The interdisciplinary paradigm that has evolved for the systematic discovery of *Conus* venom peptides, if applied more broadly, should prove fruitful for generally exploring chemical diversity from the animal biodiversity that surrounds us.

Therapeutic Applications of Conus Peptides

Six different *Conus* peptides have reached human clinical trials (Table 1); a significant milestone was the approval of a *Conus* peptide as a commercial drug for intractable pain by the Food and Administration in December 2004. The commercial product, Prialt (generically called ziconitide) is identical to the natural peptide produced by the magician's cone snail, *Conus magus*, originally designated ω-conotoxin MVIIA (4–7). This is one of a class of peptides from fish-hunting cone snail venoms exhibiting high specificity for the N-type calcium channel ($Ca_V2.2$). Another peptide that acts by the same mechanism, ω-conotoxin GVIA from *Conus geographus*, is the most widely used *Conus* peptide in neuroscience, with ~2000 research papers in the literature using this compound as a pharmacological tool (3).

A high proportion of the first conopeptides characterized have reached clinical development; of the first 30 peptides purified from *Conus* venoms, 10% reached at least Phase 1 human clinical trials. A significant fraction of these *Conus* peptides in development are potential therapeutics for pain (see Table 1). Although pharmaceutical companies have mostly focused on opioids for treating severe pain, the analgesic *Conus* venom peptides in development work through five distinct mechanisms, of which none are opioid-based. One peptide, κ-PVIIA, a K^+ channel blocker has been effective in decreasing cardiac damage in animal models of myocardial infarction (8, 9).

The diversity of analgesic conopeptides in Table 1 illustrates an important application of *Conus* peptides: identifying new pharmacological mechanisms. The N-type calcium channel had not been identified as a drug target for severe pain until ω-conotoxins were shown to be analgesic. Subsequently, small molecule drug candidates targeting N-type calcium channels were developed (10, 11); some are now undergoing human clinical trials. Thus, even those *Conus* peptides not directly developed as therapeutics can be extremely valuable for identifying potential molecular targets for novel drugs.

Gene Superfamilies Encoding *Conus* Peptides Rapidly Diversify

Conus venom peptides are being rapidly identified by using molecular cloning to deduce amino acid sequences. The continuously expanding data base provides an ever stronger case for extremely rapid diversification of genes encoding *Conus* peptides; each species has its own repertoire of 100–200 different venom peptides, distinct from that of any other species. Thus, living *Conus* are likely to express >70,000 different peptides in their venoms (3).

Most of this peptide diversity is generated by the greatly accelerated evolution of a few gene superfamilies; the nine superfamilies described to date are shown in Table 2; each *Conus* peptide gene encodes a precursor that typically has an N-terminal signal sequence of ~25 amino acids, an intervening "pro" region (~20–40 amino acids, depending on the gene superfamily), and at the C-terminal end, the mature toxin (10–40 amino acids for most superfamilies) in single copy (4). This rather conventional prepropeptide organization masks a striking and unconventional evolutionary pattern. Comparisons between superfamily genes from different *Conus* species reveal an extraordinary juxtaposition; signal sequences are conserved to an unprecedented degree but mature toxin regions are hypermutated (see Fig. 1). PCR primers designed from the conserved sequences make discovery of additional peptides in a superfamily using PCR straightforward. The predicted peptides can be chemically synthesized; for *Conus* peptides in the 10–25 amino acid size range, this is generally straightforward. One potential problem is the high frequency of post-translational modification in some *Conus* peptide families. The post-translational modification of *Conus* peptides was recently reviewed (12); more progress is needed to accurately predict post-translational modification *a priori* from cloned sequences.

The mechanisms that underlie conopeptide gene hypermutation have not been elucidated. Although there is strong diversifying selection, the pattern and frequency of sequence

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TABLE 1
Conus venom peptides with therapeutic potential

Molecular target	*Conus* peptide	Therapeutic application	Company	Development stage reached	Ref.[a]
N-type calcium channel ($Ca_v2.2$)	ω-MVIIA (Prialt; zicontide)	Pain	Elan (Neurex)	Approved by FDA 12/04	5
	ω-CVID (AM336)	Pain	Amrad	Phase 1	25
Neurotensin receptor	Contulakin-G (CGX-1160)	Pain	Cognetix	Phase 1	26
Norepinephrine transporter	χ-MrIA (derivative) (Xen-2174)	Pain	Xenome	Phase 1	27
Nicotinic receptors	α-Vc1.1 (ACV-1)	Pain	Metabolic	Phase 1	28
NMDA[b] receptors	Conantokin-G (CGX-1007)	Epilepsy; pain	Cognetix	Phase 1	29
K^+ channels (Kv1 subfamily)	κ-PVIIA (CGX-1051)	Myocardial infarction	Cognetix	Pre-clinical	8
Na^+ channels	μO-MrVIB (CGX-1002)	Pain	Cognetix	Pre-clinical	30

[a] See supplemental material for sequences and structures.
[b] *N*-Methyl-D-aspartate.

TABLE 2
Conus venom peptide gene superfamilies

Superfamily (Ref.)	Cys pattern(s) (designated framework no.)		Pharmacological family[a]	Target[a]
A (14)	CC-C-C	(I/II)	α	Nicotinic receptors
			ρ	α-Adrenergic receptors
A (16)	CC-C-C-C-C	(IV)	αA	Nicotinic receptors
			κA	K^+ channels?
M (15)	CC-C-C-CC	(III)	μ	Na^+ channels
			κM	K^+ channels
			ψ	Nicotinic receptors
O (3)	C-C-CC-C-C	(VI/VII)	ω	Ca^{2+} channels
			κ	K^+ channels
			δ	Na^+ channels
T (31)	CC——CC	(V)	None defined	Unknown
T (32)	CC——CXOC[b]		χ	Catecholamine transporter
S (33, 34)	C-C-C-C-C-C-C—C-C-C	(VIII)	σ	Serotonin ($5HT_3$) receptor
			αS	Nicotinic receptors
P (35)	C-C-C-C-C-C	(IX)	None defined	Unknown
I_1/I_2 (36)	C-C-CC-CC-C-C	(XI)	None defined	K^+ channels
J (37)	C-C-C-C	(XIV)	None defined	K^+ channels?

[a] Adapted from Ref. 4; the individual conopeptide families and their targets are described in that review, which cites the primary literature.
[b] This Cys pattern was referred to as Framework X or as Framework I by different groups (22, 38).

changes suggest special mutational/recombination mechanisms targeting mature toxin regions preferentially (involvement of a mutagenic DNA polymerase has been specifically suggested) (13, 14). Notable genetic events that generated major groups of peptides in the A-superfamily have been described (14). In contrast to the mature peptide regions, other elements in *Conus* peptide genes are much more conserved (including some intronic regions). Signal sequences, usually the least conserved element of a secreted polypeptide, are so extremely conserved that unusual genetic events (such as gene conversion) seem likely to play a role in maintaining signal sequence conservation.

Peptides of the same superfamily generally share a characteristic arrangement of Cys residues in the mature toxin region (the "Cys pattern"); each Cys pattern usually corresponds to a specific disulfide framework. However, in two gene superfamilies, each with a conserved Cys pattern, the disulfide connectivity changed as spacing of amino acids between Cys residues changed (15, 16). Thus, although peptides in a gene superfamily usually have similar structures, groups of peptides with new disulfide scaffolds can occasionally evolve within a superfamily. How small peptides with multiple Cys residues are folded such that only one disulfide framework is formed *in vivo* is a fundamental issue not yet satisfactorily addressed; if oxidative folding is carried out *in vitro*, multiple disulfide isomers are generated; the peptide with the native scaffold must be purified from other folding isomers. Preliminary indications suggest that the "Pro" region of *Conus* peptide precursors contributes at least indirectly to facilitate correct folding (17, 18).

Rapid Gene Superfamily Sequence Divergence Leads to Functional Diversification

Conus peptide superfamilies have functionally differentiated; consequently, different peptides in the same superfamily may have different classes of physiological targets. Some examples are shown in Table 2; the M-superfamily includes the μ-conopeptide family (Na^+ channel blockers), the κM-conopeptide family (K^+ channel blockers), and the ψ-conopeptide family (non-competitive nicotinic receptor antagonists). These groups of peptides are genetically and structurally related with similar Cys patterns, disulfide scaffolds, and three-dimensional structures, but they have functionally diverged. In contrast, ligand sites of peptides *in the same family* are expected to be homologous (*i.e.* μ-conopeptide binding sites on various Na^+ channel subtypes).

However, within a single *Conus* peptide family, individual peptides may differ in targeting selectivity. α-Conopeptides are nicotinic receptor antagonists, but different α-conopeptides may target different receptor subtypes. Thus, a *Conus* peptide family can provide a ligand panel selective for different molecular isoforms of a general target class. An important corollary is that it is possible to change targeting specificity of *Conus* peptides by appropriate amino acid substitutions (for a specific example, see Ref. 19).

Peptides that belong to different families may play analogous functional roles in different lineages of *Conus* species. Three different peptide families targeted to the K_V1 subfamily of voltage-gated K^+ channels, the κ-conotoxins, the conkunitzins

Mature peptide sequence

Phase 1: Peptide from Venom	Phase 2A: α-family members (Pionoconus)	Phase 2B: Non-Pionoconus α-peptides
MI GRCCHPACGKNYSC[1]	Mn1.4 NGRCCHPACAKYFSCGR[1] Cn1.1 NGRCCHPACGKHFSC[1] Sm1.1 GRGRCCHPACGPNYSC[1] SIA YCCHPACGKNFDC[1] MII GCCSNPVCHLEHSNLC[2]	AuIB GCCSYPPCFATNPDC[3] PIA RDPCCSNPVCTVHNPQIC[4] RgIA GCCSDPRCRYRCR[5] Br1.4 ITCCTRGTCAQHC ImI GCCSDPRCAWRC[6] ImII ACCSDRRCRWRC[7]

Open reading frame (peptide precursor)

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Cn1.1  MFTVFLLVVLTTTVVSFPSDSASDVR DDEAKDERSDMYKSK-R-NGR CC -HPA C GKHFS-- C GR
Sm1.1  MFTVFLLVVLATTVVSFPSDRASDGR DDEAKDERSDMHESGRKGRGR CC -HPA C GPNYS-- C GR
MII    MFTVFLLVVLATTVVSFPSDRASDGR NAAANDKASDVITLALK---G CC SNPV C HLEHSNL C GRRR
PIA    MFTVFLLVVLATTVGSFTLDRASDGR DAAANDKATDLIALTAR-RDP CC SNPV C TVHNPQI C G
AuIB   MFTVFLLVVLATTVVSFTSDRASDGR ----KDAASGLIALTMK---G CC SYPP C FATNPD- C GRRR
```

Phylogenetic Tree (16s)

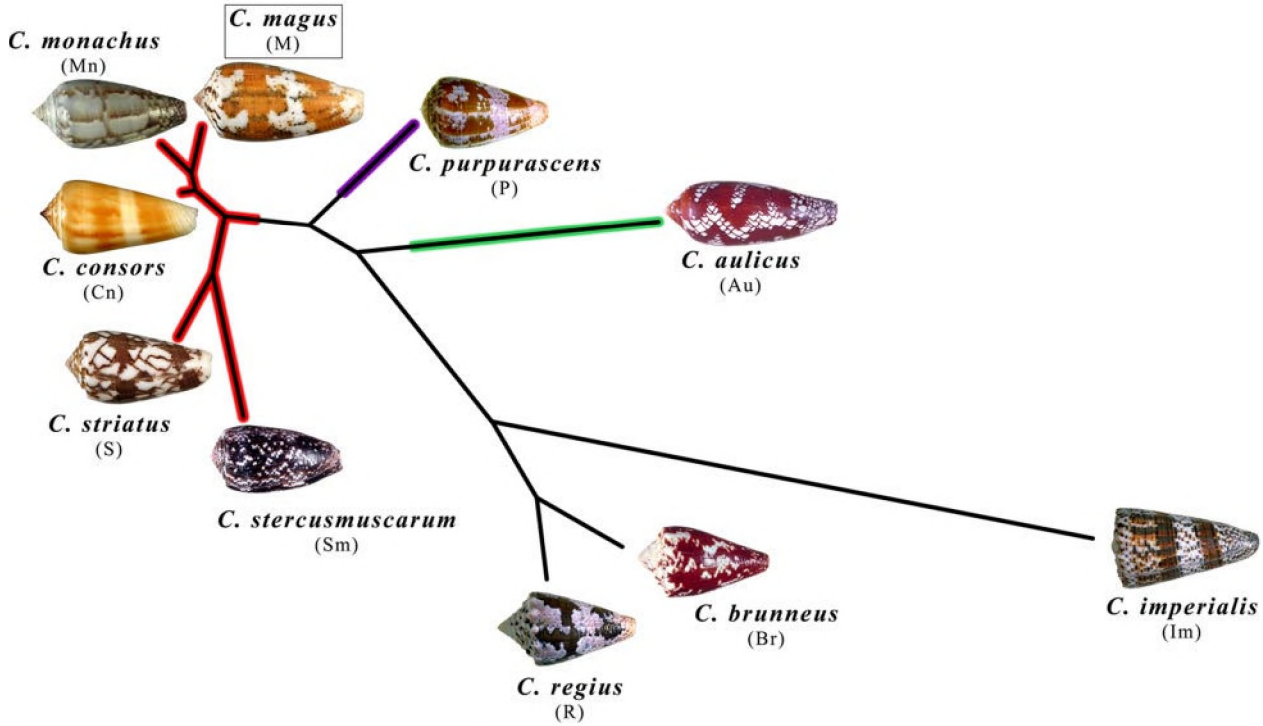

FIGURE 1. **Incorporating phylogeny into biochemical discovery.** The figure shows a panel of α-conopeptides targeted to different nicotinic receptor subtypes (*top*), the prepropeptide precursor sequences of some of these α-conopeptides (*middle panel*), and the *Conus* species these were derived from (*bottom panel*). *Top*, a venom peptide targeting muscle nicotinic receptors, α-MI, a member of the α-conopeptide family, was purified from *C. magus* venom (Phase 1). cDNA libraries from species in *Pionoconus*, in the same subgenus as *C. magus* (*C. monachus, C. consors, C. stercusmuscarum, C. striatus*), yielded other family members (Mn1.6, Cn1.1, Sm1.1, and SIA) with the same muscle subtype selectivity (shown as *superscript 1*) as α-MI; in the *C. magus* cDNA library, a second family member, α-MII, was identified with different subtype selectivity (neuronal α3β2, shown as *superscript 2*) (Phase 2A). Analysis of species less closely related to *C. magus* and not in *Pionoconus* (*C. regius, C. brunneus, C. imperialis, C. purpurascens, C. aulicus*) uncovered α-family members that were more divergent (Phase 2B). Although these peptides also target nicotinic receptors, they have different subtype selectivity (AuIB:α3β4 (*superscript 3*), PIA:α6β2 (*superscript 4*), RgIA:α9α10 (*superscript 5*), Iml, α7+α3β2 (*superscript 6*), αImII,α7 (*superscript 7*)). *Middle panel*, complete amino acid sequences of some α-conopeptide family precursors are shown; mature peptide sequences are at the C terminus after the *arrow*, the site of proteolytic cleavage releasing the mature peptide toxin. The conserved N-terminal region as well as the pattern of Cys residues in the mature peptide (both *boxed*) clearly identifies these peptides as members of the A-conopeptide superfamily. All members of the superfamily targeted to the nicotinic receptor family and exhibiting the characteristic Cys pattern (CC---C---C) belong to the α family. Note that the first two sequences encode peptides that belong to the α3/5 subfamily, whereas the bottom three are members of two other subfamilies. *Bottom panel*, phylogenetic tree. The relationship of *C. magus* to other species is shown in the phylogenetic tree based on 16 S sequences. The *red* branches show species in the subgenus *Pionoconus*, and the *purple* branch is a fish hunter in *Chelyconus*. The *green* branch shows a mollusk hunter (*C. aulicus*). The *uncolored* branches are worm-hunting species that specialize in amphinomid polychaetes; these express α-conopeptides in the α4/3 subfamily.

(14), and the κM conotoxins (18), are completely unrelated genetically and structurally. Three different clades of fish-hunting cone snails each evolved peptides belonging to the three unrelated gene families for parallel physiological purposes (blocking the K_V1 subfamily of K^+ channels, as part of the "excitotoxic shock" strategy to rapidly immobilize fish prey) (20, 21). Although different clades of *Conus* species utilize different conopeptide families to inhibit the K_V1 subfamily of K^+ channels, all of these clades use the same family, the δ-conopeptides, to inhibit Na^+ channel inactivation (20). Thus some conopeptide families are found in a single clade comprising a few species, whereas others appear to be found in all ~700 *Conus* species.

Systematic Discovery and Characterization of Conus Peptides Using Phylogenetics

In recent years, *Conus* peptide discovery has become more efficient by integrating phylogenetics into the discovery strategy. A prime example of systematic discovery using such a phylogenetically informed strategy has been the α-conopepide family peptides, which target nicotinic receptors. Some *Conus* species from which α-conotoxins have been characterized are shown in Fig. 1; major α-conopeptides from different species are shown. The first group analyzed were fish-hunting *Conus* belonging to one clade, the subgenus *Pionoconus*; the major α-conopeptides found belong to the same subfamily of α-conopeptides (the α3/5 subfamily) that specifically target the muscle nicotinic receptor subtype. Scanning different sequences of α3/5 subfamily peptides provides structure-function information; the pattern of amino acid conservation identifies the likely functionally important residues.

By analyzing species less related to *C. magus* (in clades other than *Pionoconus*), other α-conopeptide subfamilies were identified. The worm-hunting species *Conus imperialis, regius*, and *brunneus* express peptides in the α4/3 subfamily, primarily targeted to homomeric nicotinic receptor subtypes (*i.e.* α7, α9, α10) (22–24) instead of to the muscle subtype. Thus, a systematic sweep of biodiversity can be used to identify groups of peptides in the same family, which have diverged in target selectivity. Closely related species yield functionally homologous peptides; more divergent species express family members likely to have different (but related) molecular targets. This discovery strategy has provided the neuroscience community with a panel of α-conopeptides diagnostic for various nicotinic receptor subtypes.

Perspectives

Every *Conus* species has a repertoire of 100–200 conopeptides with essentially no overlap between different species. The venom peptide families rapidly diversify. The conopeptide repertoire of a particular cone snail species mediates interactions between that species and its prey, predators, and competitors (2). Thus venom peptide genes are sculpted by natural selection to the ecological singularities of the individual species.

In any megadiverse group of related animals such as the cone snails, most of the genome would be expected to be largely conserved if the different species within the group were compared. In contrast, genes responsible for mediating interactions with other animals in the environment, such as the conopeptide genes, have to diversify rapidly because each species has its own ecological niche. The spectrum of biotic interactions of a species differs from that of all others; this is the perspective that rationalizes why a distinctive complement of venom peptides is found in each of the ~700 different *Conus* species. Thus, the evolutionary history of such genes would be expected to be strikingly different from most of the rest of the genome, a hypothesis strongly supported by the accumulating data on *Conus* peptide genes.

The rapidly diversifying genes encoding *Conus* peptides are, we believe, not at all unique in their evolutionary behavior; they are merely the first major class of such genes to have been extensively characterized from a substantial number of related animal species. The rationale above for why venom peptide genes rapidly diversify leads to the expectation that in general, genes whose products act on other animals in the environment will evolve extremely rapidly. For other megadiverse animal lineages, similarly rapidly diversifying genes should be a major genetic foundation for generating biodiversity.

The molecular genetic revolution has been based on a few model organisms (*Escherichia coli*, yeast, *Caenorhabditis elegans*, *Drosophila*, zebra fish, and mice), and these models provide a powerful common framework for understanding genes that encode central physiological functions. *Conus* peptide genes are qualitatively different; their gene products are exogenously targeted. Although the endogenous physiology of different species in *Conus* is likely to be closely similar, exogenous interactions with prey, predators, and competitors differ sharply between species. The genes whose ultimate gene products act on the physiology of a different organism will thus be expected to comprise a part of the genome greatly accelerated in its rate of evolution. It is useful to distinguish this rapidly diversifying sector of the genome from the general class of "endogenously targeted" genes by the term "exogenome."

To understand biodiversity, particular attention needs to be paid to the exogenome; understanding how evolution of the exogenome is accelerated may hold the key to understanding speciation at a molecular level. The work on *Conus* peptide genes has revealed some esoteric and remarkable features including an unprecedented rate of the divergence of mature peptide regions juxtaposed with an almost total conservation of signal sequences. Will these features also be characteristic of genes in the exogenomes of other megadiverse taxa? The unusual features of conopeptide genes may well be generally diagnostic of the exogenome, consisting of genes that diversify rapidly as speciation occurs. Identifying genes comprising the exogenome, determining whether these systematically differ in their organization from other genes, and learning how to rapidly deduce and express or synthesize the gene products that they encode should coalesce into an organized genomic subdiscipline, exogenomics, that has enormous potential for future biomedical/pharmacological discovery.

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