



Pruning nature: Biodiversity-derived discovery of novel sodium channel blocking conotoxins from *Conus bullatus*

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ABSTRACT

Described herein is a general approach to identify novel compounds using the biodiversity of a megadiverse group of animals; specifically, the phylogenetic lineage of the venomous gastropods that belong to the genus *Conus* ("cone snails"). Cone snail biodiversity was exploited to identify three new μ -conotoxins, BullIA, BullIB and BullIC, encoded by the fish-hunting species *Conus bullatus*. BullIA, BullIB and BullIC are strikingly divergent in their amino acid composition compared to previous μ -conotoxins known to target the voltage-gated Na channel skeletal muscle subtype $\text{Na}_v1.4$. Our preliminary results indicate that BullIB and BullIC are potent inhibitors of $\text{Na}_v1.4$ (average block $\sim 96\%$, at a $1 \mu\text{M}$ concentration of peptide), displaying a very slow off-rate not seen in previously characterized μ -conotoxins that block $\text{Na}_v1.4$. In addition, the three new *C. bullatus* μ -conopeptides help to define a new branch of the M-superfamily of conotoxins, namely M-5. The exogene strategy used to discover these Na channel-inhibiting peptides was based on both understanding the phylogeny of *Conus*, as well as the molecular genetics of venom μ -conotoxin peptides previously shown to generally target voltage-gated Na channels. The discovery of BullIA, BullIB and BullIC Na channel blockers expands the diversity of ligands useful in determining the structure–activity relationship of voltage-gated sodium channels.

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1. Introduction

A significant accomplishment of molecular neuroscience has been identifying membrane macromolecules that underlie nervous system function. An extraordinarily conserved set of such proteins, including ion channels and neurotransmitter receptors, are present in all nervous

systems from *Caenorhabditis elegans* to humans. These molecules are encoded by a few gene superfamilies (such as the voltage-gated and ligand-gated ion channel superfamilies), with each superfamily comprising several families (e.g., Na channels responsible for action potentials are in a family that belongs to the voltage-gated ion channel superfamily) (Goldin et al., 2000; Hille, 2001; Ogata and Ohishi, 2002).

Every ion channel/receptor family, in turn, comprises multiple subtypes or isoforms. In mammals, nine isoforms of the major α -subunit of voltage-gated Na channels, each encoded by a different gene, have been characterized (Catterall, 1992, 2000). Despite the breakthroughs in identifying the molecular components of the nervous

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system, delineating the functions of each has been a challenge. The standard technology for investigating function is knockout mice; thus, knockouts of individual Na channel subtypes have been made (Planells-Cases et al., 2000; Amaya et al., 2006; Woodruff-Pak et al., 2006). However, the difficulty and expense of maintaining the mice, the fact that some knockouts do not thrive and the unpredictability of whether knocking out of one subtype will result in a compensatory, and possibly ectopic, over-expression of another makes the development of alternative methods for investigating function highly desirable.

An attractive alternative would be to use pharmacological methods; the limiting factor is that highly subtype-selective ligands to study function are seldom available, particularly for ion channels. For the voltage-gated sodium channels, the most widely used pharmacological tool is tetrodotoxin, an alkaloid whose best known source is puffer fish (Yotsu et al., 1987). Of the nine Na channel subtypes in mammals, six are tetrodotoxin-sensitive and three are insensitive (Catterall et al., 2005). Thus, at the present time, pharmacological characterization using tetrodotoxin clearly does not have the resolution provided by knockout mice. More selective ligands for voltage-gated Na channels need to be developed.

One biological system being broadly exploited to generate ligands with greater subtype selectivity for ion channels are the small peptides found in cone snail venoms (Norton and Pallaghy, 1998). There is a remarkable molecular diversity in each venom; the 700 species of cone snails each have ~100 different peptide toxins, and rapid interspecific divergence has generated ~70,000 different peptides. Like their ion channel targets, cone snail peptides are encoded by gene superfamilies that in turn comprise families, and each family of conopeptides generally targets a corresponding ion channel or receptor family (Terlau and Olivera, 2004). For example, the M superfamily includes the μ -conopeptide family, delineated with the Cys framework –CC–C–C–CC–, each member of which targets one or more of the nine subtypes of voltage-gated sodium channels. Hypermutation of *Conus* peptide genes occurred as cone snails speciated, and they are examples of “exogenes,” those genes responsible for mediating biotic interactions between organisms. Exogenes are characteristically extremely rapidly diverging; the accelerated evolution of exogenes expressed in *Conus* venom ducts has generated a biochemical and pharmacological diversity that can be exploited to develop highly subtype-selective ligands for ion channels and receptors (Olivera, 2006; Ellison and Olivera, 2007; Olivera and Teichert, 2007).

Cone snails can be grouped into discrete clades. Our laboratory has analyzed the molecular phylogeny of cone snails; the resultant phylogenetic information has been incorporated into a general strategy for the discovery of novel classes of peptides. This “exogenome” strategy was used effectively to obtain highly subtype-selective *Conus* peptides that discriminate between nicotinic acetylcholine receptor subtypes (Olivera, 2006). The analysis of *Conus* peptide exogene families, when combined with informed phylogenetics, makes screening the enormous pharmacological resource afforded by cone snail venom peptides far more efficient.

In this paper, exogene analysis and phylogenetics were used to identify three new μ -conotoxins, BullIIA, BullIIB and BullIIC. BullIIA, BullIIB and BullIIC have a significantly different amino acid composition from previous μ -conotoxins known to target the voltage-gated Na channel subtype $\text{Na}_v1.4$, yet are extremely potent inhibitors of this subtype. BullIIA, BullIIB and BullIIC from *Conus bullatus*, are apart of a novel class of μ -conopeptides discovered from a newly defined clade of fish-hunting cone snails, the *Textilia* clade. In addition, *C. bullatus* peptides help define a new branch of the M-superfamily of conotoxins, namely M-5. The discovery of these three novel Na channel blockers expands the diversity of ligands useful in determining the structure–activity relationship of voltage-gated sodium channels.

2. Materials and methods

2.1. Cloning and identification of *C. bullatus* μ -conotoxins from cDNA

Dissection of *C. bullatus* venom ducts, construction of cDNA, and PCR amplification and cloning of μ -conotoxins were as previously described (West et al., 2002; Azam et al., 2005). Briefly, venom ducts were dissected from living snails and stored at -80°C . Venom duct tissue was homogenized and RNA extracted using Trizol reagent according to the manufacturer's standard protocol (Trizol Total RNA Isolation – Life Technologies/Gibco BRL, Grand Island, NY). cDNA was prepared by reverse transcription of RNA isolated from *Conus* venom ducts as previously described (Jacobsen et al., 1998). The resulting cDNA served as a template for PCR amplification using primers based on conserved 5'- and 3'-UTR regions of previously isolated μ -conotoxin genes: Forward primer: 5' CAA GA(AG) GGA TCG ATA GCA GTT C 3'. Reverse primer: 5' ACT GCA ATC (AG)TT TTA CTT ATT C 3'. Analysis of the cDNA sequences identified the open reading frames encoding the complete precursor protein for μ -conotoxins. Aligned nucleotide sequences of BullIIA, BullIIB and BullIIC, with primer sequence region highlighted are shown in Supplementary Fig. 1 (S1). DNA Sequences for BullIIA, BullIIB, and BullIIC were deposited into GenBank, accession numbers FJ240165–167.

2.2. PCR amplification of 16S rRNA gene segment of mtDNA for *C. bullatus* species and 16S sequence analysis

16S rRNA was amplified as previously described (Espiritu et al., 2001). Nucleic acid sequences were aligned manually using MEGA version 3.1 (Kumar et al., 2004). The phylogenetic tree was created from two independent runs using the software program MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). A total of 8,000,000 trees were made in each run, 80,000 of which were saved. A total of 1500 of each of those 80,000 were also discarded as burn-in. Each run had four chains (one cold and three heated). The two independent runs were combined into a single tree where branches were retained if they were found in 50% or more of those trees not discarded. The standard deviation after 8,000,000 generations was 2.836×10^{-3} . A general time reversible (GTR) model

was used, with the rate variation of some sites kept invariable and the remaining rates drawn from a gamma distribution.

2.3. Chemical synthesis and two-step oxidative folding of μ -conotoxins

Synthetic conotoxins were produced using methods identical to those described previously (Bulaj et al., 2005, 2006). Briefly, the peptides were synthesized on solid support using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. The peptides were cleaved from the resin by a 3- to 4-h treatment with reagent K (trifluoroacetic acid (TFA)/water/ethanedithiol/phenol/thioanisole; 82.5/5/2.5/7.5/5 by volume). The peptides were subsequently filtered and precipitated with cold methyl-*tert*-butyl ether (MTBE). The linear peptides were purified by reversed phase HPLC using a semi-preparative C₁₈ Vydac column (218TP510, 10 mm $\times$ 250 mm), over a linear gradient of 10–30% ACN in 40 min and the fractions were detected at 220 nm. Peptide was further characterized using ESI-MS (Micromass Quattro II mass spectrometer) at Mass Spectrometry and Proteomic Core Facility of the University of Utah.

To facilitate the folding of *C. bullatus* peptides with the native Cys connectivity (Cys1–Cys4, Cys2–Cys5 and Cys3–Cys6), peptides containing acetamidomethyl (Acm)-protection of Cys3 and Cys6 were constructed. A two-step folding protocol was used to produce the correctly folded species of BullIA, BullIB, and BullIC. HPLC comparison of traditional oxidation and folding protocols, in which none of the Cys are protected, and the two-step approach indicated the two-step approach garnered a better yield of the final product (see [Supplementary Fig. 2](#)). Formation of the first two disulfide bridges (Cys1–Cys4 and Cys2–Cys5) was accomplished by oxidation of linear peptides in buffered solution (0.1 M Tris–HCl, pH 7.5) containing 1 mM EDTA, 1 mM oxidized glutathione, 1 mM reduced glutathione, and 1 M NaCl. Folding was quenched by acidification with formic acid (8% final concentration). The main oxidation product was purified from the folding mixture by semi-preparative HPLC, and further characterized using ESI-MS. Formation of the final disulfide bridge was accomplished by a simultaneous removal of Acm-protecting groups, and subsequent oxidation with 1 mM iodine containing 25% ACN in H₂O. Iodine oxidation was quenched after 10 min at room temperature by drop wise addition of 1 M ascorbic acid. Final purification of fully folded peptide was accomplished by semi-preparative HPLC using conditions described above and also characterized using mass spectrometry.

2.4. Electrophysiology of cloned rat Na_v1.4 expressed in *Xenopus* oocytes

The cDNA clone for Na_v1.4 was kindly provided by Dr. Al Goldin. The cDNA was linearized with NotI and transcribed with T7 RNA polymerase using the mMessage mMachine RNA transcription kit from Ambion (Austin, TX) to generate capped cRNA. The cRNA was then purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA).

Oocytes were each injected with about 15 ng of cRNA in about 30 nl of distilled water and incubated at 16 °C in ND96 (composition in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH $\sim$ 7.3) supplemented with Pen/Strep, Septra and Amikacin for 1–5 days. To record sodium currents from a given oocyte, it was placed in a cylindrical well (4 mm diameter, volume $\sim$ 50 μ l) in a wafer made of Sylgard (Dow Corning, Midland, MI). The oocyte was impaled with glass microelectrodes filled with 3 M KCl (0.1–0.5 M Ω) and two-electrode voltage clamped using a Warner OC-725C amplifier (Warner Instruments, Hamden, CT). The membrane potential was held at -80 mV, and sodium channels were activated by a 50 ms step to -10 mV applied every 30 s. The current signals were low-pass filtered at 3 kHz, digitized at a sampling frequency of 10 kHz, and leak-subtracted by a P/6 protocol using in-house software written in LabVIEW (National Instruments, Austin, Texas). The oocyte chamber was intermittently perfused with ND96 using a motorized syringe (Cavro XL3000, Tecan Systems, San Jose, CA) or peristaltic pump (Rainin, Woburn, MA). To apply toxin, the perfusion was halted, 5 μ L of toxin solution (at 10 times of the final concentration) was applied to the 50 μ L bath, and the bath manually stirred for about 5 s by gently aspirating and expelling $\sim$ 5 μ L of bath fluid several times with a micropipette. Recordings were conducted at room temperature.

3. Results

3.1. Using phylogeny for μ -conotoxin discovery

μ -Conotoxins are a family of Na channel antagonists widely distributed across *Conus*. Several groups have reported a molecular phylogeny for the cone snails (Duda and Palumbi, 1999; Espiritu et al., 2001; Duda and Kohn, 2005) a section of the phylogenetic tree is shown in [Fig. 1](#). New *Conus* included in the tree are: *C. bullatus*, *Conus cervus* and *Conus dusaveli*, all fish-hunting species from relatively deep water (see [Fig. 2](#)). This species group is referred to as the *Textilia* clade; the type species of *Textilia* (Swainson, 1840) is the bubble cone *C. bullatus*. As shown in [Fig. 1](#), previously characterized μ -conotoxins belong to three different clades of fish-hunting *Conus* (*Gastridium*, *Chelyconus* and *Pionoconus*), each represented by a discrete branch on the phylogenetic tree. The skeletal muscle (Na_v1.4)-specific peptides, μ -GIIIA (Cruz et al., 1985) and μ -PIIIA (Shon et al., 1998), came from snails in two different clades, *Gastridium* and *Chelyconus*. Notably, the μ -cono-peptides from species in the *Pionoconus* clade were previously shown to target neuronal Na channel subtypes (Zhang et al., 2006).

Representative species from 10 clades of *Conus* from which μ -conotoxins had not been characterized, including the newly defined *Textilia* clade were chosen for analysis. We took advantage of the fact that μ -conotoxins belong to the M-superfamily (Corpuz et al., 2005). Potential μ -conotoxin sequences were obtained by PCR using primers based on conserved sequence elements of M-superfamily genes. A large number of M-superfamily sequences (>100) were obtained; the analysis of these sequences revealed putative new sequences that belong to the μ -conotoxin family. Of these, those from the representative species of

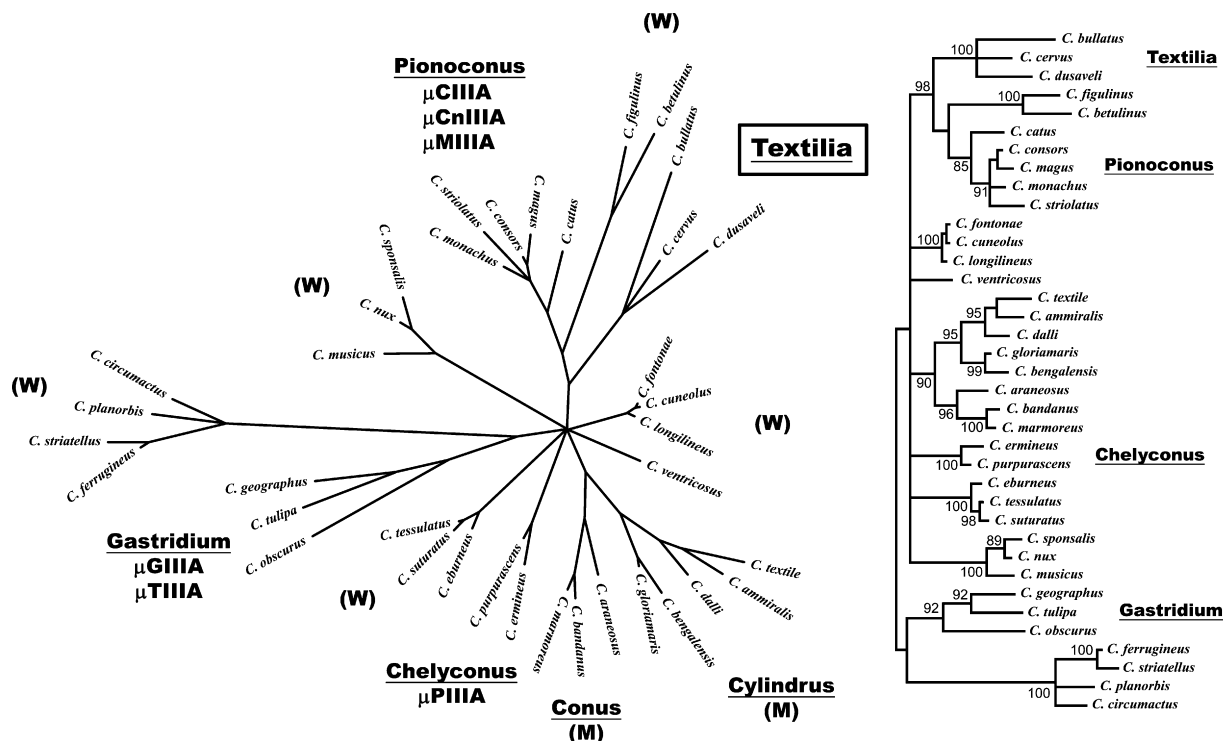


Fig. 1. Phylogenetic tree of *Conus*. The phylogenetic tree was constructed based on 16S mitochondrial DNA sequences as described in Section 2. Not all clades of *Conus* are shown; however, all lineages with previously characterized μ -conotoxins are indicated, including the *Gastridium*, *Chelyconus* and *Pionoconus* clades. μ -Conotoxins from the species shown in the phylogenetic tree are indicated below the name of the clade. Three species not previously included in an analysis of the molecular phylogeny of *Conus*, *C. bullatus*, *C. cervus* and *C. dusavelli*, form a distinct branch defining the *Textilia* clade. There are two clades that comprise all known mollusk-hunting species, the subgenera *Conus* and *Cylindrus*, and their branches are labeled "(M)". The branches labeled "(W)" are various worm-hunting clades, the taxonomic status of these is presently being evaluated by Kohn and co-workers. The same tree is shown on the right, but with confidence values above 80% indicated; note that the four clades that are labeled are all branches on the tree with high confidence values.

the *Textilia* clade, *C. bullatus*, seemed the most distinctive and promising.

Three putative μ -conotoxin cDNA clones with unique structural features were identified from the cDNA library from *C. bullatus* venom ducts (see Section 2). The predicted prepropeptide sequences are shown in Table 1; they are typical of precursors in the μ -conotoxin family. The striking signal sequence conservation of the μ -cono-peptide precursors is evident by comparison of *C. bullatus* sequences with previously determined μ -cono-peptide precursor sequences derived from *Conus* species in other clades, which are also shown in Table 1. The post-translational processing of a μ -cono-peptide (prepropeptide) is well defined and predictable (Corpus et al., 2005); the precursors are proteolytically converted to the mature toxin at defined sites (–KR in the specific prepropeptide sequences shown).

It should be noted that of the total number, 21, of *C. bullatus* cDNA M-superfamily clones analyzed, 10 encoded μ -BullIIA and a polymorphic variant, two encoded μ -BullIIB and one encoded μ -BullIIC.

3.2. Analysis of the translated μ -conotoxin sequences from *C. bullatus*

The predicted mature toxins from the *C. bullatus* cDNA sequences are compared to those of previously

characterized μ -conotoxins in Tables 2 and 3. The three *C. bullatus* peptides, BullIIA, BullIIB and BullIIC clearly define a distinctive class of μ -conotoxins, different from those discovered so far from the other phylogenetic lineages of *Conus*. The three mature μ -conotoxin sequences obtained from *C. bullatus* differ from the overall consensus sequence of all the μ -conotoxins from *Pionoconus*, *Gastridium* and *Chelyconus* shown in Table 2, which is: $X_3CCX_5CX_4CX_{4-5}CC$, where X is an amino acid other than Cys. In contrast, the consensus for the three *C. bullatus* peptides is: $X_4CCX_{5-7}CX_3CX_5CC$. Thus, in addition to AA substitutions, *C. bullatus* peptides as a class have unique features: all three peptides have four AA before the first cysteine, a variable length of 5–7 AA in the first loop (between the second and third Cys residues), and only three amino acids in the second loop (between the third and fourth Cys residues), instead of the four AA present in the other μ -conotoxins (Tables 2 and 3). In addition, neither BullIIA, BullIIB, nor BullIIC have a glutamine residue on the amino terminus that is often posttranslationally modified to pyroglutamate, unlike several of the μ -conotoxins in the *Gastridium* and *Chelyconus* clades (Table 2).

3.3. Chemical synthesis of *C. bullatus* μ -conotoxins

We chemically synthesized and folded all *C. bullatus* peptides, μ -conotoxins BullIIA, BullIIB, and BullIIC, and tested



Fig. 2. Fish-hunting *Conus* species belonging to the *Textilia* clade. Shown are shells of three different *Conus* species (top, *C. bullatus*; middle, *C. cervus*; bottom, *C. dusaveli*) that form a well-defined branch on the phylogentic tree (See Fig. 1); the taxonomic designation for this group is *Textilia* (Swainson, 1840) with the type species being the bubble cone, *C. bullatus*. These species have not been extensively analyzed previously largely because of their deep-water habitat; it has been a challenge to collect species such as *C. cervus* and *C. dusaveli*, considered by collectors as among the 50 rarest seashells (a good shell specimen of *C. cervus* sell for \$400–\$1200).

their activities. The chemical synthesis and the folding protocols are described in Section 2. A two-step protocol using analogues containing acetamidomethyl (Acm)-protection of Cys3 and Cys6 was designed to facilitate folding of *C. bullatus* μ -conotoxins into the form with native Cys connectivity (Cys1–Cys4, Cys2–Cys5 and Cys3–Cys6). Fig. 3 depicts a representative HPLC profile of the two-step oxidative folding of BullIB. The first folding step with

glutathione produced a major species that was purified by preparative HPLC, and subsequently oxidized with iodine. The final oxidation step yielded a single major product. The same methodology was used to produce BullIA and BullIC. In each case, the chemical identity of the final product was verified by mass spectrometry.

3.4. Evaluation of BullIA, BullIB, and BullIC activity on $Na_v1.4$ channels

The activities of the three *C. bullatus* peptides were assessed on $Na_v1.4$ expressed in *Xenopus* oocytes (see Section 2) and compared to the activities of a representative set of μ -conotoxins, PIIIA, GIIIA, and KIIIA (Zhang et al., 2007). PIIIA and GIIIA belong to the *Chelyconus* and *Gastroidium* clades, and were selected for their remarkable divergence in amino acid sequence compared with BullIA, BullIB and BullIC; PIIIA and GIIIA contain postrationally modified residues on the amino-terminal and in the first and third Cys-loop (Table 2). KIIIA is similar to the *C. bullatus* μ -conotoxins; however, it has significant structural differences at the N-terminus and first Cys loop (Table 3). All *C. bullatus* μ -conotoxins were tested at the same concentration (1 μ M) and under identical conditions, representative recordings are shown in Fig. 4. BullIA, BullIB and BullIC inhibited most, if not all, of $Na_v1.4$ sodium current. As shown in the bar graph of Fig. 5, *C. bullatus* peptides BullIB and BullIC are potent antagonists of $Na_v1.4$ with an average block of $\sim 96\%$. KIIIA and BullIA inhibit $Na_v1.4$ similarly ($\sim 87\%$ block). PIIIA and GIIIA activities on $Na_v1.4$ were determined from IC_{50} values published by Safo and colleagues (Safo et al., 2000). The calculated block of $Na_v1.4$ by PIIIA (96% block) and GIIIA (98% block) is similar to that by BullIB and BullIC.

The block of $Na_v1.4$ by BullIA was reversible ($k_{off} = 0.021 \pm 0.006 \text{ min}^{-1}$, $N = 3$ oocytes), and the current largely recovered in an hour. In contrast, BullIB is very slowly reversible (Fig. 6) as is BullIC (not shown), and recovery from block in each case was too slow to determine k_{off} values in the time frame of the experiments. GIIIA is the best-studied μ -conotoxin, and its reversibility ($k_{off} = 0.14 \pm 0.015 \text{ min}^{-1}$, $N = 3$ oocytes) is also illustrated in Fig. 6 for comparison. The detailed electrophysiological characterization of BullIA, BullIB and BullIC will be described elsewhere.

Table 1
Conus bullatus μ -conotoxins precursors

Conotoxin	Signal sequence	Propeptide	Mature toxin
BullIA	MSKLGVLLTICLLLFPLFALPQDGDOPADRPAERMQDDISSEONSLLEKR/VTORCC	---	XGKREC-GRWCRDHSRCCGRR
BullIB	MSKLGVLLTICLLLFPLFALPQDGDOPADRPAERMQDDISSEONPLLEKR/VGERCC	--	XNGKRGCGRWCRDHSRCCGRR
BullIC	MSKLGVLLTICLLLFPLFALPQDGDQPADRPAERMQDDLSSEQHPLFEKR/IVORCC	NKNGKRGCG	SRWCRDHSRCCGRR
CnIIIA	MSKLGVLLTICLLLFPLIALPMDGDOPADRPAERMQDDISSQHPDFDKR/	GRCC	---DVPNACSGRWCRDHAQCCG
CnIIIB	MSKLGVLLTICLLLFPLFALPLDGDQPADRPAERMQDDISSEKHPLFDKR/	QGCC	---GEPNLCFTWRNCRNARCCRQQ
GIIA	MSKLGVLLTICLLLFPLIALPMDGDEPANRPVERMQDNISSEQYPLFEKR/	RQCC	---TPPKCKDRQC-KPQRCCAGR
PIIA	MSKLGVLLTICLLLFPIIALPMDGDQPADRLAERMQDNISSEHP-FEKR/	QRLCC	---GFPKSCRSRQC-KPHRCCGR

Table 2
Predicted mature toxins from *i*-Conus cDNA sequences

Species	μ -Conotoxin	Mature toxin Sequence	Reference
<i>Textilia</i>			
<i>C. bullatus</i>	BullIA	VTDR CC KGKRE CGRW CRDHSR CC #	This work
<i>C. bullatus</i>	BullIB	VGER CC KNGKRG CGRW CRDHSR CC #	This work
<i>C. bullatus</i>	BullIC	IVDR CC NKNGKRG CSRW CRDHSR CC #	This work
<i>Gastrium</i>			
<i>Conus geographus</i>	GIIA	RD CC TOOKK CKDRQ CKOQR CCA #	Cruz et al. (1985)
<i>C. geographus</i>	GIIB	RD CC TOORK CKDRR CKOMK CCA #	Cruz et al. (1985)
<i>Conus tulipa</i>	TIIA	RH GC CKGOK GCS SRE CROQH CC#	Lewis et al. (2007)
<i>Chelyconus</i>			
<i>C. purpurascens</i>	PIIA	ZRL CC GFOKS CS SRQ CKOHR CCG#	Shon et al. (1998)
<i>Pionoconus</i>			
<i>Conus consors</i>	CnIIIA	GR CC DVPNA CS GRW CRDHA QCC#	Zhang et al. (2006)
<i>C. consors</i>	CnIIIB	ZG CC GEPNL CF TRW CR NNAR CCR QQ^	Zhang et al. (2006)
<i>Conus magus</i>	MIIA	ZG CC NVPNG CS GRW CRDHA QCC#	Zhang et al. (2006)
<i>Conus catus</i>	CIIIA	GR CC GPN GCS SRW CKD HAR CC #	Zhang et al. (2006)

Z and O represent post-translationally modified residues pyroglutamate and hydroxyproline, respectively. #C-terminal amidation, ^C-terminal free acid.

4. Discussion

A noteworthy aspect of this work is the discovery of a distinctive group of μ -conotoxins that are expressed in the venom of *Conus* species in the *Textilia* clade. One reason why the fish-hunting cone snails in the *Textilia* clade have not been extensively analyzed is their deep-water habitat; it has been a challenge to collect species in this clade such as *C. cervus* and *C. dusavehi*, considered by collectors as among the 50 rarest seashells. However, *C. bullatus* is more accessible, and we were able to gradually accumulate enough venom ducts to carry out an extensive analysis of cDNA clones.

Ultimately, we were able to identify and characterize three μ -conotoxins from the *Textilia* clade of *Conus* that antagonizes $\text{Na}_v1.4$. This particular tetrodotoxin-sensitive isoform of voltage-gated Na channels has previously been shown to be a target for μ -conotoxins, however, the strikingly divergent sequences of *C. bullatus* peptides from previously characterized μ -conotoxins and their potency in inhibiting $\text{Na}_v1.4$, make them novel tools for probing the structure–activity relations of voltage-gated sodium channels. A full investigation of the activity of *C. bullatus* peptides on other subtypes of Na channels is currently underway. The discovery of *C. bullatus* μ -conotoxins was accelerated by use of an exogene strategy to identify evolutionarily relevant *Conus* species.

Table 3
New M-5 superfamily of μ -conotoxins

Species	μ -Conotoxin	Mature toxin Sequence
<i>C. bullatus</i>	BullIA	VTDR CC KGKRE CGRW CRDHSR CC #
<i>C. bullatus</i>	BullIB	VGER CC KNGKRG CGRW CRDHSR CC #
<i>C. bullatus</i>	BullIC	IVDR CC NKNGKRG CSRW CRDHSR CC #
<i>C. consors</i>	CnIIIA	GR CC DVPNA CS GRW CRDHA QCC#
<i>C. consors</i>	CnIIIB	ZG CC GEPNL CF TRW CR NNAR CCR QQ^
<i>C. magus</i>	MIIIA	ZG CC NVPNG CS GRW CRDHA QCC#
<i>C. catus</i>	CIIIA	GR CC GPN GCS SRW CKD HAR CC #
<i>C. kinoshitai</i>	KIIIA	CC N CS SKW CRD HSR CC #
<i>C. striatus</i>	SIIIA	ZN CC NGG CSS KW CRD HAR CC #
<i>C. stercus-muscarum</i>	SmlIIIA	ZR CC NGRRG CSS RW CRD HSR CC #

It is significant that the first three peptides from the *Textilia* clade characterized differ greatly in their amino acid composition compared to previous antagonists of the $\text{Na}_v1.4$ subtype. All *C. bullatus* peptides can be differentiated from previously characterized μ -conotoxins by their longer N-terminal extension (4 versus 0–3 AA) and fewer residues

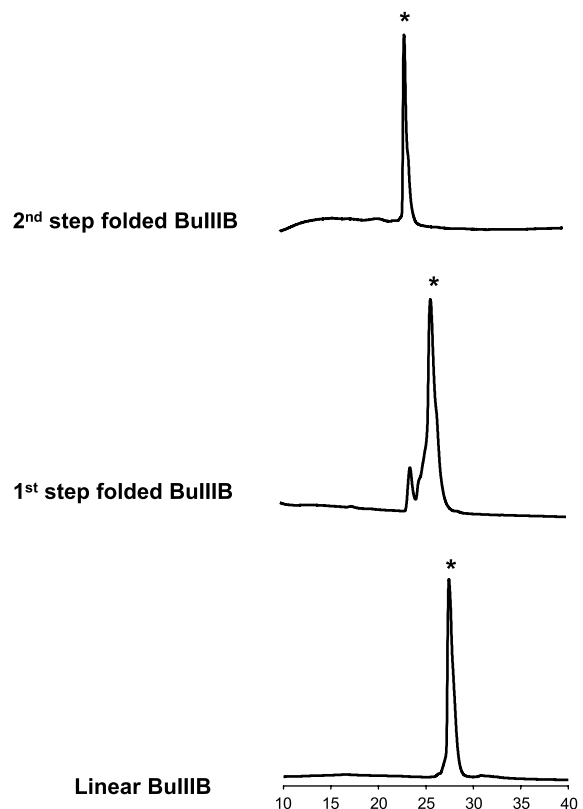


Fig. 3. Two-step oxidative folding of *C. bullatus* μ -conotoxin BullIB. RP-HPLC elution profiles of all intermediates obtained during the folding of linear BullIB. The peptide was folded by the two-step procedure described in Section 2. BullIA and BullIC were prepared similarly. The linear and correctly folded products are indicated with an asterisk (*).

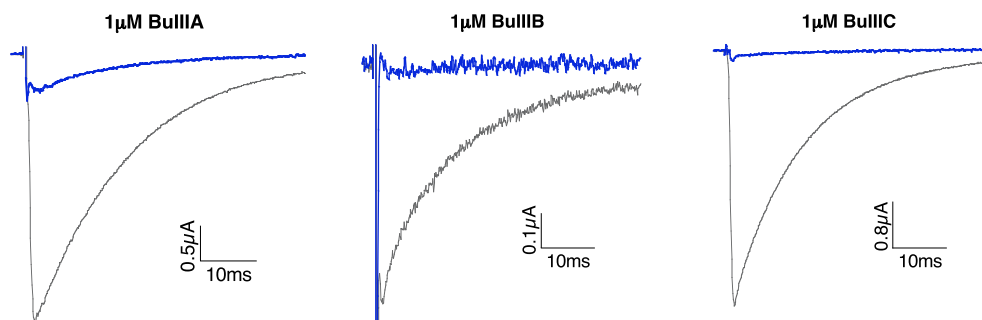


Fig. 4. Block of $\text{Na}_v1.4$ by μ -conotoxins BullIA, BullIB, BullIC. Voltage-clamp recordings were performed on *Xenopus* oocytes expressing $\text{Na}_v1.4$ as described in Section 2. Representative sodium currents recorded in the absence of toxin (control traces, gray) and following ~ 30 min exposure to $1 \mu\text{M}$ BullIA, BullIB, and BullIC (black traces). Each trace represents the average of five responses.

in the second cys-flanked loop (3 instead of 4). Previously, M-superfamily conotoxins were divided into four branches based on the number of residues in the third Cys-flanked loop, namely, M-1, M-2, M-3, and M-4 (Corpus et al., 2005). BullIA, BullIB, and BullIC, together with SmIIIA (West et al., 2002), SIIIA (Bulaj et al., 2005), KIIIA (Bulaj et al., 2005) and the μ -conotoxins identified in the *Pionoconus* clade in Table 2, comprise a new branch of the M-superfamily, M-5, in which there are five residues in the third Cys-loop of the mature toxin sequence (Table 3). M-5 μ -conotoxins have a significant degree of sequence homology in the third Cys loop, but differ as described above at the amino termini and in the first and second Cys-flanked loops. The ratio of residues in the second versus third loops, which we refer to as M-3/5 for BullIA, BullIB, and BullIC, and M-4/5 for the other μ -conotoxins in the M-5 family may account, in part, for the differences in their activity on $\text{Na}_v1.4$. Absent from the M-5 superfamily is hydroxyproline, which are found in the second and third loops in the M-4 family members GIIIA, GIIIB, TIIIA, and PIIIA in Table 2.

Recent comparative functional analyses of the structure–activity relationship of M-4 μ -conotoxin GIIIA (Choudhary et al., 2007) and M-5 μ -conotoxin KIIIA (Zhang et al., 2007) have led to the identification of specific residues involved in high affinity interactions with $\text{Na}_v1.4$. Using pair-wise alanine mutations and double mutant cycle analysis, Choudhary and colleagues identified novel toxin–channel interactions involving residues K9, K11, K16, and R19 in GIIIA. Of these, only R19, the homolog of which is R24 in BullIC, and R13 in GIIIA, which was previously identified as critical for activity (Sato et al., 1991; Dudley et al., 1995; French et al., 1996; Hui et al., 2002), the homolog of which is R17 in BullIC, are conserved for all M-5 μ -conotoxins except MIIIA and CnIIIA where the residue in the equivalent location of GIIIA's R19 is glutamine (Table 2). The residues homologous to those believed to be important for KIIIA inhibition of $\text{Na}_v1.4$ (W8, R10, D11, H12 and R14), are highly conserved in BullIA, BullIB, and BullIC (Table 3). However, our results indicate these shared features, by themselves, are not sufficient to explain the higher affinity for $\text{Na}_v1.4$ of *C. bullatus* peptides BullIB and BullIC compared to KIIIA (Fig. 5). BullIA and KIIIA have similar activities on $\text{Na}_v1.4$ (Fig. 5), including being easily reversible (not illustrated). In contrast, BullIB and BullIC are more potent inhibitors of $\text{Na}_v1.4$, like PIIIA and GIIIA (Fig. 5).

However, PIIIA and GIIIA are readily reversible inhibitors (Safo et al., 2000), while BullIB and BullIC are almost irreversible (Fig. 6), both displaying a uniquely slow off-rate. The fact that BullIB and BullIC block $\text{Na}_v1.4$ with about the same affinity as PIIIA and GIIIA given the significant differences in their amino acid composition is of importance. In frog neuromuscular preparations, PIIIA specifically blocks skeletal muscle Na channels irreversibly (Shon et al., 1998), and this property can be exploited to study synaptic potentials without confounding muscle action potentials. In view of their irreversibility, BullIB and BullIC may likewise be useful for studies of mammalian muscle preparations. Additionally, *C. bullatus* μ -conotoxins lack post-translationally modified residues such as pyroglutamate and hydroxyproline found in PIIIA and GIIIA, making them easier to chemically synthesize.

One possible hypothesis to be tested to explain the difference in activities of *C. bullatus* μ -conotoxins, BullIA, BullIB, and BullIC is that the specific combination of

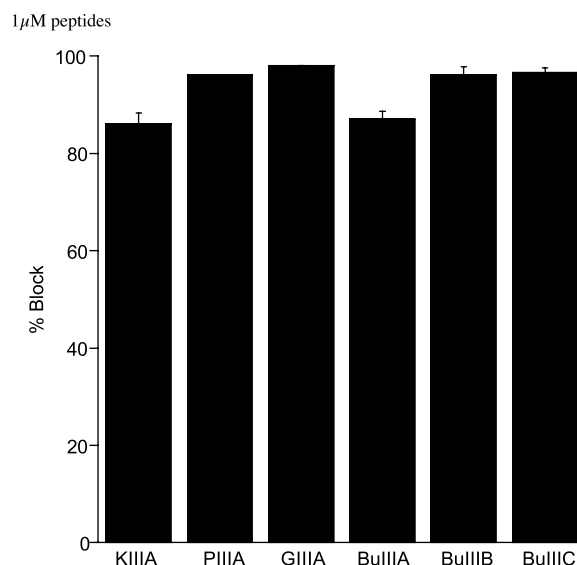


Fig. 5. Comparison of the block of $\text{Na}_v1.4$ produced by $1 \mu\text{M}$ μ -conotoxins from *C. bullatus* and other *Conus* species. PIIIA is from *Conus purpurascens* (*Chelyconus* clade), GIIIA is from *C. geographus* (*Gastriidum* clade), and KIIIA is from *Conus kinoshitai*, whose phylogeny has not been determined. Values for PIIIA and GIIIA were calculated from IC_{50} values given in Safo et al. (2000).

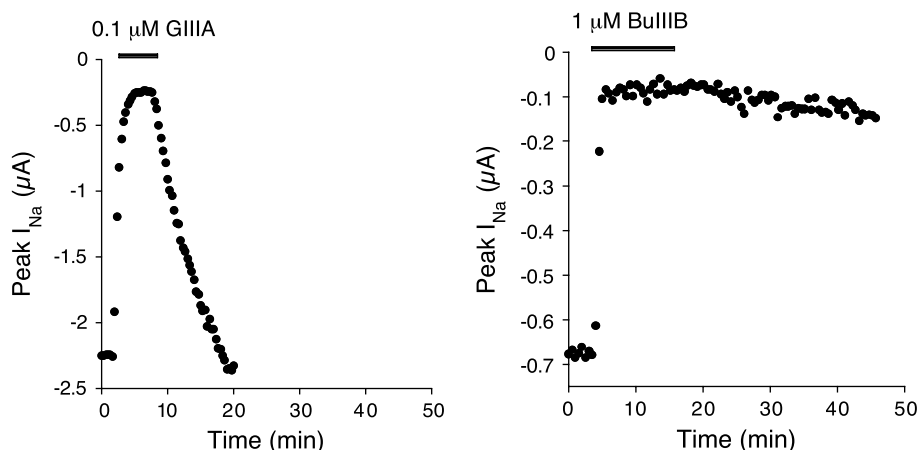


Fig. 6. Time course of block and recovery of $\text{Na}_v1.4$ following exposure to GIIIA and BullIB. The bar at the top of each graph represents when the peptide was present at the indicated concentration. GIIIA is readily reversible whereas BullIB is relatively irreversible.

additional residues on the N-terminus, and the absence of a fourth residue in the second loop are critical for determining higher affinity of μ -conotoxins for $\text{Na}_v1.4$. Experiments along these lines are currently being investigated.

In addition to the intrinsic importance of ligands specific for $\text{Na}_v1.4$, this work pioneers a new approach for the discovery of subtype specific ligands in the *Conus* venom peptide system. In principle, the same general strategy can be applied to discovery from any phylogenetic lineage of animals. Some features of this discovery strategy may seem almost trivial: for example, it is critical to identify the tissue producing the compounds that the organism uses to interact with other organisms. For a venomous animal such as *Conus*, this is unambiguously the venom duct. Directly analyzing the source tissue makes the requisite molecular biology vastly more facile.

A second requirement of the discovery strategy is to elucidate the molecular genetics of the ligands of interest; in this case, the relevant gene family is the μ -cono-peptides, which belong to the M-superfamily. This family and superfamily are sufficiently well defined to be accessible for analysis by PCR (Corpuz et al., 2005). A third component of the discovery strategy is knowledge of the phylogenetics (as illustrated in Fig. 1). Different M-superfamily sequences were collected from over 10 different clades of *Conus*, and the bioinformatics analysis of the sequences suggested that the *C. bullatus* μ -conotoxin sequences from the *Textilia* clade were a sufficiently distinctive class so that further characterization, i.e., chemical synthesis and folding, was justified. Thus, a chemical synthetic capacity, and the ability to fold the linear conopeptides efficiently are additional technical requirements for the type of conopeptide discovery detailed in this paper. The final requirement is routine access to a sensitive assay – in this case, cloned $\text{Na}_v1.4$ expressed in *Xenopus* oocytes. This work highlights the exogene strategy as an effective method for identifying novel peptides with unique structural and functional properties. The three μ -conotoxins from *C. bullatus*, BullIA, BullIB, and BullIC, contain novel structural determinants that confer noteworthy activity for skeletal muscle subtype $\text{Na}_v1.4$. These ligands could be used in the continuing effort

to accurately define the features of the $\text{Na}_v1.4$ pharmacophore and thereby facilitate the design of highly subtype-specific ligands that target $\text{Na}_v1.4$.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.toxicon.2008.10.017.

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