A Novel Inhibitor of α9α10 Nicotinic Acetylcholine Receptors from Conus vexillum Delineates a New Conotoxin Superfamily

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Abstract

Conotoxins (CTxs) selectively target a range of ion channels and receptors, making them widely used tools for probing nervous system function. Conotoxins have been previously grouped into superfamilies according to signal sequence and into families based on their cysteine framework and biological target. Here we describe the cloning and characterization of a new conotoxin, from Conus vexillum, named αβ-conotoxin VxXXIVA. The peptide does not belong to any previously described conotoxin superfamily and its arrangement of Cys residues is unique among conopeptides. Moreover, in contrast to previously characterized conopeptide toxins, which are expressed initially as prepropeptide precursors with a signal sequence, a “pro” region, and the toxin-encoding region, the precursor sequence of αβ-VxXXIVA lacks a “pro” region. The predicted 40-residue mature peptide, which contains four Cys, was synthesized in each of the three possible disulfide arrangements. Investigation of the mechanism of action of αβ-VxXXIVA revealed that the peptide is a nicotinic acetylcholine receptor (nAChR) antagonist with greatest potency against the α9α10 subtype. 1H nuclear magnetic resonance (NMR) spectra indicated that all three αβ-VxXXIVA isomers were poorly structured in aqueous solution. This was consistent with circular dichroism (CD) results which showed that the peptides were unstructured in buffer, but adopted partially helical conformations in aqueous trifluoroethanol (TFE) solution. The α9α10 nAChR is an important target for the development of analgesics and cancer chemotherapeutics, and αβ-VxXXIVA represents a novel ligand with which to probe the structure and function of this protein.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels used throughout the animal kingdom for neurotransmission. These receptors are assembled from α, β, γ, δ and/or ε subunits to form multiple receptor subtypes with distinct pharmacological properties [1]. Elucidation of the precise structure and function of various nAChRs is challenging owing to the scarcity of ligands selective for specific receptor subtypes. In an effort to address this, we have systematically examined components of the venoms of carnivorous cone snails for selective nAChR-targeted ligands.

Mollusks of the genus Conus are comprised of >700 species. These marine snails hunt primarily polychaete worms, molluscs or fish. Each cone species produces a cocktail of >100 different compounds that enables prey capture. Despite extensive work, the vast majority of these compounds remains uncharacterized.

Conopeptides are produced in the venom duct of Conus and used offensively to immobilize prey. Their potency and selectivity for various ion channels and receptors have made them excellent pharmacological probes and drug leads [2–3]. The term conotoxin is used to describe the subset of Conus peptides that are rich in Cys residues. Conotoxins are synthesized initially as precursor proteins that are subsequently processed into the mature toxin. Previously characterized Conus peptides have been grouped into gene superfamilies based on similarities in their precursor signal sequences [4]. Within each superfamily, the toxins are grouped according to cysteine frameworks that influence their final three-dimensional structure. The toxins are also grouped according to receptor or ion channel target into pharmacological families. Within a given family of conotoxins there is, characteristically, hypervariation in non-Cys residues, which is believed to enable selective action on a given target subtype. Post-translational

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modification or chemical synthetic modification provides further diversity [3–6].

Toxins characterized to date can be classified into one of 17 superfamilies (see Table 1) [46–61]. The current study characterizes a new toxin from the worm-hunting Conus vexillum, with a unique Cys framework. As the precursor sequence does not align with any of the previously-reported gene superfamilies, this peptide represents a first-in-class compound (see Table S1). Total chemical synthesis was carried out to enable pharmacological and structural characterization of this novel toxin. The peptide acts as an antagonist of nicotinic acetylcholine receptors, with greatest structural characterization of this novel toxin. The peptide represents a first-in-class compound (see Table S1).

Materials and Methods

Ethics Statement

No specific permits were required for the described field studies. No specific permissions were required for Tanmen Qionghai, Hainan Province, China, which is not privately-owned or protected in any way. The field studies did not involve endangered or protected species.

Table 1. Protein precursor sequences of Conus gene superfamilies [46–61].

| Super-family Peptide | Precursor Sequence (Signal, N-terminal pro-regions, | Mature peptide and | C-terminal pro-regions | Reference |
|----------------------|----------------------------------------------------|--------------------|------------------------|-----------|
| B                    | αB-VxXXIVA  | METLTLWRRASCLLLVVLGLSRLIRLLG | **| V1C1EL5QGTPNKLFLRPPCCGKGPSAFRHSRCVYTVQISRE | This Study  |
| A                    | α-LITa     | MGRMMRFIMFMLVLATTVTFTSDRAKLNAMADDNENKA |                |                        | 46,47,48  |
| D                    | αD-VxXXB   | MPKLAVVVLILPL5SYAAGQAVGIDWGNLBAR |                |                        | 49,50     |
| I1                   | Alx1A      | MKLCATFLVLVPLVLTGKSSRSLLGLGVR | **| RTCS                   | 51        |
| I2                   | BeTX       | MMIFRTVSGCLLIVLFNVLTPSA | | CREA0TGCENDS | 52        |
| I3                   | Ca11.3     | MKLVIAVIMILSLSTGAEMSDNASHABARLDRLLSP |                |                        | 53        |
| J                    | Fe14.1     | MPSRSWTVCCCLLWMLSMQLSVTPGSTAQLSHRTA | J | SPSTDC0MACRTGNGNHPYSCNPQ | 31        |
| K                    | im23a      | MIRRVMTLFVLVMTAASAGDALTEARIOYPQGTGAA | ECYSWNVKODLSDWCCDCLKHADMMMPAAGCC | | 8        |
| L                    | C14.1a     | MNVTYMDLLVTLLMTPMTIGWNTTPQNMGGPQVRQDAG | NVLNDHGQFR | | 54        |
| M                    | ψ-PrIIIE   | MLKSSLVLLTCLTIPALVDGGQADPRHPVERADDNQYIS | EKHPFXXA | | 51        |
| O1                   | SO3        | MKTCMTHVAYVLLTCATILRSADDQSTQHWTI3DTTX | SMSTR | CAAKGRGCSBAYNCTCQSGS | G | 51        |
| O2                   | BeB54      | MKLTLVIALVAVMETSOIAQRGIDQREKQARLNNLS65 | K | STAESWEGCGWSYCVSDWCCGEGCTTRYECL | 31        |
| O3                   | CaFr179    | MSGLGMVYTLVILLTFEMASHQDGERAQTORDAIVR | RSLL55 | TVITEECCCEEDEEHHKCNNTTNGPSGCARLF | G | 57        |
| P                    | GmIVXA     | MHL5SALS3AAMLFLALGNFVWSSGQTITRDQNGQ | TDNARNLNOEWNPSLSM56 | SCNNSGCSHSCDASHICTTFRGCGAVN | G | 58        |
| S                    | αS-GVIIIA  | MMSKGAGFMVLLFLTTNLST3QGQDVQRKTR7KDSDYF | ALARDRR | GCTRTEGQPKCICGCTNSISGRCGNYQVNHPSWGCGAC | G | 59        |
| T                    | VvOB       | VLLLAAISAPSDAVQPKTDVDPAILHONAFQDALNQ | R | CCQTYTMYCCQ | G | 59        |
| V                    | VvXVA      | MMPVVLILLLSLIRACDAGAVQDGPSASLLTGNH | LPPVR | DCITCAAGEECGQCTCPWQDNCSCPEW | G | 60        |
| Y                    | CaXVIIA    | MOKATVAVLALLPLISTAQDEGSQAQDARQEDRAT | | | 61        |
cDNA Sequencing

Total RNA was extracted from individual ducts and purified as described previously [11]. Venom duct cDNA library construction followed the kit manufacturer’s suggested protocol. Briefly, the first-strand cDNA was synthesized with the SMART IV Oligo-nucleotide and transcriptase. Full-length, double-stranded (ds) cDNA (SMART cDNA) was generated by long-distance PCR. SMART cDNA was ligated into the Sfi I predigested pDNA-R-LIB vector. The signal and mature peptide sequences of the conotoxin precursors were predicted using online ProP 1.0 Server [12].

Peptide Synthesis

The linear peptide was assembled by solid-phase methodology on an ABI 433A peptide synthesizer using FastMoc (N-(9-fluorenyl) methoxycarbonyl) chemistry and standard side-chain protection, except for cysteine residues. Cys residues of the three possible isomers were protected in pairs with either S-trityl on Cys3 and Cys19 (designated βB-VxXXIVA [1,2]), Cys3 and Cys20 (designated βB-VxXXIVA [1,3]), Cys19 and Cys20 (designated βB-VxXXIVA [1,4]) or S-acetamidomethyl on Cys20 and Cys32, Cys19 and Cys32, Cys3 and Cys32, respectively. The peptides were removed from a solid support by treatment with reagent K(TFA / water / ethanedithiol / phenol / thioanisole; 90:5:2.3:7.5:5v/v/v/v/v) respectively. The peptides were removed from a solid support by treatment with reagent K(TFA / water / ethanedithiol / phenol / thioanisole; 90:5:2.3:7.5:5v/v/v/v/v). The released peptide was precipitated and washed three times with cold ether. A two-step oxidation protocol was used to fold the peptides selectively, as described previously [13]. Briefly, the disulfide bridge between Cys3 and Cys19, Cys3 and Cys20, or Cys19 and Cys20, respectively, was closed by dripping the peptide into an equal volume of 20 mM potassium ferricyanide, 0.1 M Tris, pH 7.5. The solution was allowed to react for 45 min, and the monocyclic peptide was purified by reverse-phase HPLC. Simultaneous removal of the S-acetamidomethyl groups and closure of the disulfide bridge between Cys20 and Cys32, Cys19 and Cys32, or Cys3 and Cys32, respectively, was carried out by iodine oxidation as follows: the monocyclic peptide in HPLC eluent was dripped into an equal volume of iodine (10 mM) in H2O:TFA:acetonitrile (74:2:4 by volume) and allowed to react for 10 min. The reaction was terminated by the addition of ascorbic acid, diluted 10-fold with 0.1% TFA, and the bicyclic peptide was purified by HPLC on a reversed-phase C18 Vydac column using a linear gradient of 20–60% B60 in 40 min. Solvent B was 60% ACN, 0.092% TFA, and H2O; Solvent A 0.1% TFA in H2O. Peptide concentration was measured using absorbance at 280 nm, and calculated using the Beer-Lambert equation and a calculated molar extinction coefficient of 3040 cm⁻¹ M⁻¹.

cRNA Preparation and Injection

Capped cRNA for the various subunits were made using the mMessage mMachine in vitro transcription kit (Ambion) following linearization of the plasmid. The cRNA was purified using the Qiagen RNAeasy kit. The concentration of cRNA was determined by absorbance at 260 nm. Oocytes were injected within one day of harvesting and recordings were made 1–4 days post-injection.

Voltage-clamp Recording

Oocytes were voltage-clamped and exposed to ACh and peptide as described previously [14]. Briefly, the oocyte chamber consisting of a cylindrical well (~30 μl in volume) was gravity perfused at a rate of ~2 ml/min with ND96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, pH 7.1–7.5) containing 0.1 mg/ml BSA. The Ba²⁺-ND96 had 1.8 mM BaCl₂ in place of CaCl₂. The membrane potential of the oocyte was clamped at ~70 mV. The oocyte was subjected once a minute to a 1 s pulse of 100 μM ACh. In the case of the α9α10 and muscle α1βδε subtypes, there is a 1 s pulse of 10 μM Ach, and for the α7 subtype a 200 μM ACh pulse. For toxin concentrations ≥10 μM, once a stable baseline was achieved, either ND96 alone or ND-96 containing conotoxin was applied manually for 5 min prior to the addition of the agonist. All recordings were done at room temperature (~22°C).

Data Analysis

The average of five control responses just preceding a test response was used to normalize the test response to obtain “% response.” Each data point of a dose-response curve represents the average ± S.E. of at least three oocytes. The dose-response data were fit to the equation, % response = 100/[(toxin)/IC50]nH, where nH is the Hill coefficient, by non-linear regression analysis using GraphPad Prism (GraphPad Software).

NMR Spectroscopy

1H NMR spectra were recorded on βB-VxXXIVA isomers at a concentration of ~330 μM in 20 mM phosphate/10% H2O buffer at pH ~5.8. The 1D spectra were acquired on a Bruker Avance 600 MHz NMR spectrometer equipped with cryogenic probe fitted with a z axis gradient. The NMR spectra were collected at 5°C using the excitation sculpting pulse sequence [15]. Spectra were acquired over 4K data points with 64 scans and a 1H spectral width of 14 ppm. All spectra were processed in TOPSPIN (version 3.0) and referenced to the water resonance.

Circular Dichroism Analysis

βB-VxXXIVA isomers were dissolved in 20 mM phosphate buffer (pH 5.9) and CD spectra were recorded on a Jasco-815 spectropolarimeter at a concentration of 43 μM at 20°C. Spectra were collected at 0.05 nm intervals over the wavelength range 260–195 nm in a 10 mm pathlength cuvette. Three scans were collected and averaged for each peptide sample with scanning rate of 100 nm/min⁻¹. The spectra were then smoothed using a third-order polynomial function. In order to investigate the effect of trifluoroethanol (TFE) on the conformation, CD spectra for βB-VxXXIVA [1,2] were also acquired following the addition of 10, 20, 50 and 87% TFE. The % α-helix and β-sheet content were calculated from the CD data using the CDPro program [16].

Results

Discovery and Sequence Analysis of a cDNA of the Precursor of βB-VxXXIVA

In general, conotoxins are translated initially as prepropeptide precursors [7], with proteolytic cleavage yielding the final product(s). Peptides in the same superfamily are characterized by highly conserved prepropeptide precursor sequences. This conservation has allowed direct identification of new peptides belonging to a particular superfamily by cDNA sequencing of family or superfamily genes [17]. However, a large fraction of conotoxins present in the Conus genus has yet to be sequenced and several additional families of toxins remain to be identified. Most of the cone snails investigated to date are fish- or mollusc-hunters. In an effort to discover novel conotoxin families, we examined the worm-hunting C. vexillum. Specimens were collected from the South China Sea and dissected venom ducts were used to construct a cDNA library. Approximately 50 clones from the cDNA library were chosen randomly for sequencing and inspected for previously unreported sequences. In the present study with C. vexillum, several members of the previously characterized α- and 0-
Superfamilies were identified. In addition, however, an unusual precursor sequence was noted (Fig. 1, Table 1, GenBank accession number JX297421). A sequence similarity search detected no homology with precursors of the known superfamilies of conotoxins [7] (Table S1). The sequence was analyzed with DNAstar software and online ProP 1.0 Server [12], which indicated a 28-residue signal sequence followed by a previously unreported 40-residue mature toxin (Table 2 and see also Table S2 for sequence alignment). For other conotoxins, the encoding cDNA has a characteristic three-region organization, including a signal sequence, a “pro” region, and the toxin-encoding region [7,18]. The generation of the mature toxin requires proteolytic cleavage of the N-terminal prepro-region of the precursor. In contrast to previously characterized conopeptide toxins, the precursor of αB-VxXXIVA has no “pro” region. The putative proteolytic processing site between prepropeptide and mature region for conotoxins is usually a basic amino acid (K or R). In contrast, the predicted mature peptide exhibited a new cysteine arrangement: Cys3-Cys19, Cys20-Cys32 (Fig. 1). The predicted mature peptide exhibited a new cysteine framework, not previously reported for conotoxins, C–CC–C (Table 2 and see also Table S2 for sequence alignment). The mature toxin region is underlined. The stop codon is indicated as *. Unlike previously reported conotoxins, there is no pro region.

Synthetic peptides with these disulfide bond arrangements were used in all subsequent studies.

**Figure 1. αB-Conotoxin VxXXIVA prepeptide and encoded toxin are shown.** A putative proteolytic processing site (G) is indicated by the arrow. The mature toxin region is underlined. The stop codon is indicated as *. Unlike previously reported conotoxins, there is no pro region. doi:10.1371/journal.pone.0054648.g001
Thus, the potency of the αβ-VxXXIVA isomers in the presence of Ba^{2+} was similar to that seen in Ca^{2+}, consistent with the toxin effect being due to blockade of the nAChR rather than blockade of the Ca^{2+}-activated Cl^{-} channel.

**NMR Studies**

The 1D 1H NMR spectra of αβ-VxXXIVA isomers in phosphate buffer at pH 5.8 show that the majority of the amide protons fall within the 8.0–9.0 ppm range (Fig. 6); the lack of chemical shift dispersion here and elsewhere in the spectrum indicates that these isomers lack any significant tertiary structure. The same was true at pH 7.0 (Fig. S1, S2, S3). NMR spectra were also acquired in the presence of 3–10 mM CaCl_{2} to ascertain whether calcium had any effect on their conformation, but no change in chemical shift dispersion was observed (Fig. S4).

**Circular Dichroism Analysis**

CD spectra were acquired on all three αβ-VxXXIVA isomers in phosphate buffer. All peptide isomers exhibited minima at around 200 nm (Fig. 7A), indicative of a random coil conformation with no α-helical and β-sheet content, and consistent with our NMR results. As TFE is known to stabilize the α-helical structure in proteins and peptides [19], CD spectra of one of the isomers (αβ-VxXXIVA [1,2]) were recorded in increasing concentrations of TFE. Upon addition of 50–85% TFE, αβ-VxXXIVA [1,2]



![Figure 3. αβ-Conotoxin VxXXIVA blocks α9α10 nAChRs. (A)](image)

**Table 2.** Mature toxin sequences of nAChR-targeted conotoxin superfamilies [62–68].

| Peptide     | Superfamily | Mass | Cysteine Residues | Sequence          | Reference |
|-------------|-------------|------|-------------------|-------------------|-----------|
| αβ-VxXXIVA  | B           | 4623 | 4                 | VRCLEKSGAQINKLFRPPCCQKGP5FAHRSHCYYYYQTSRE 40aa | This Study |
| α-AuIB      | A           | 1573 | 4                 | GCCSYPPFCATPMPDC#/ 15aa | 62        |
| α-A-DIVB    | A           | 1865 | 6                 | CCGGVDAACACPOVCNICTC#/ 19aa | 63        |
| α-C-PyKA    | T           | 3492 | 2                 | TGYIYDAKPOF5SCAGLRRGCGLPNONLRKFE#/ 32aa | 64        |
| α-D-VxXIVA  | D           | 5134 | 10                | DVQDCQVSTGKWSRCCNLVPVGPNGCCPSSHVCYVTHRGRGHC#/ 47aa | 65,66     |
| α-E-RVIIIA  | S           | 5168 | 10                | KCHFDKCKGTGVYNGK5CSCXGLH5CRCTYNIGSMKSGACACITY5 47aa | 67        |
| γ-PiIIIE    | M           | 2716 | 6                 | HODCCLGKRRYOGCSSACCC#/ 24aa | 68        |

O = hydroxyproline, X = gamma-carboxyglutamate, # = C-terminus amidation,
= C-terminus COOH.

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Figure 2. Amino acid sequence of αβ-Conotoxin VxXXIVA. Three possible isomers with different disulfide connectivities: αβ-VxXXIVA [1,2] with a disulfide connectivity I–II, III–IV; αβ-VxXXIVA [1,3] with I–III, II–IV and αβ-VxXXIVA [1,4] with I–IV, II–III.
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Figure 3. αβ-Conotoxin VxXXIVA blocks α9α10 nAChRs. (A) Xenopus oocytes expressing α9α10 nAChR were voltage clamped at −70 mV and subjected to a 1 s pulse of ACh every min as described in Materials and Methods. A representative response in a single oocyte is shown. After control responses to ACh, the oocyte was exposed to 10 μM toxin for 5 min (arrow). After the 5 min toxin application, a response to ACh was measured (a). After 1 min of toxin washout, another response to ACh was measured (b). Note that the response to ACh recovered to control level after 1 min of toxin washout. (B) Concentration response of α9α10 nAChRs exposed to the three different isomers of αβ-VxXXIVA (see Fig 2). Values shown in the graph are mean ± SEM from 3–5 separate oocytes. The IC_{50}s were: αβ-VxXXIVA [1,2], 1.2 μM (0.8–1.7 μM); αβ-VxXXIVA [1,3], 3.9 μM (2.7–5.6 μM); and αβ-VxXXIVA [1,4] > 30 μM. Hill slopes were αβ-VxXXIVA [1,2], 1.4 (0.5–2.1) and αβ-VxXXIVA [1,3, 1.3 (0.9–1.7).
doi:10.1371/journal.pone.0054648.g003
Materials and Methods

described in Materials and Methods. “C” indicates control responses to ACh. Oocytes were then exposed to 10 μM peptide for 5 min, followed by application of ACh. The peptide blocked α9α10 but not α7, α3β4 or α4β2 nAChRs.
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showed slightly increased ordered structure, as evident by the shift in the minimum towards 208 nm, with some ellipticity also developing at 222 nm (Fig. 7B). The CD data were fitted using three algorithms (CDSSTR, CONTINLL, and SELCON) in CDPro [16]. The outputs obtained from all three algorithms gave very similar values and indicated that the 2B-VxXXIVA [1,2] isomer in the presence of 87% TFE had ~ 42% α-helix, ~ 8% β-strand and ~ 50% unordered structure (including turns), whereas, in the absence of TFE it had ~ 7% α-helix, ~ 31% β-strand and ~ 62% unordered structure.

Discussion

Conotoxins are a highly specialized set of disulfide-bonded peptides that are structurally and functionally diverse. Despite this diversity, toxins identified to date may be grouped into approximately 17 gene superfamilies based on conservation of the signal sequence. Within these gene superfamilies, the mature peptides adopt one of 23 patterns of arrangement of cysteine residues. Pharmacological targets within a gene superfamily may differ. For example, in the A superfamily, there are both paralytic and excitotoxic peptides [20].

It is very likely that the previously described superfamilies and Cys frameworks represent only a small fraction of the total chemical space of conotoxins. C. vexillum inhabits waters up to 70 m deep in Hainan province of the South China Sea and feeds on eunicid worms. Here, we describe the discovery and characterization from this species of 2B-VxXXIVA, a peptide that differs in substantial aspects from previously-reported conotoxins.

The clone for 2B-VxXXIVA was obtained from random sequencing of a cDNA library prepared from venom ducts. The signal sequence of 2B-VxXXIVA does not align well with the signal sequence of other known conotoxins. Conservation of the signal sequence has previously been exploited as a means of cloning novel conotoxins from different species of cone snails [21]. The unique signal sequence of 2B-VxXXIVA explains why this novel conotoxin has not been detected previously with screening primers designed to recognize known gene superfamilies. The discovery of 2B-VxXXIVA expands the known complexity of this group of ion channel- and receptor-targeted ligands. Interestingly, the precursor for 2B-VxXXIVA is unique among conotoxins in that it lacks a pro region. The pro region of disulfide-bonded

Figure 4. αβ-Conotoxin VxXXIVA differentially blocks α9α10, α7, α3β4 and α4β2 nAChRs. nAChR subtypes were expressed as described in Materials and Methods. "C" indicates control responses to ACh. Oocytes were then exposed to 10 μM peptide for 5 min, followed by application of ACh. The peptide blocked α9α10 but not α7, α3β4 or α4β2 nAChRs.
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![Figure 4](image-url)

Table 3. IC50 and Hill slope values for block of rat nAChR subtypes by αβ-Conotoxin VxXXIVA [1,2].

| nAChR subtype | IC50 | IC50 C.I. | Hill Slope | Hill Slope C.I. |
|---------------|------|----------|------------|----------------|
| Rat α2β2      | 23.4 μM | 17.3–31.5 μM | 1.1 | 0.7–1.5 |
| Rat α2β4      | >30 μM   | –                                  | – | – |
| Rat α3β2      | >30 μM   | –                                  | – | – |
| Rat α4β2      | >30 μM   | –                                  | – | – |
| Rat α6/α3β2β3 | 12.2 μM  | 10.0–14.9 μM   | 1.2 | 0.9–1.7 |
| Rat α6/α3β2β4 | 30.1 μM  | 18.8–48.0 μM   | 1.0 | 0.5–1.5 |
| Rat α7        | >30 μM   | –                                  | – | – |
| Rat α9α10     | 1.2 μM   | 0.8–1.7 μM    | 1.4 | 0.5–2.1 |
| Mouse α1β1γδ  | 6.6 μM   | 5.1–8.5 μM    | 1.2 | 0.8–1.6 |

C.I., 95% confidence interval.
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Figure 5. Concentration-response of αβ-Conotoxin VxXXIVA on α9α10 nAChR in the presence of Ba++ . Equimolar Ba++ was substituted for Ca++, in the perfusion solution as described in Materials and Methods, to prevent activation of endogenous Xenopus Ca++ activated Cl− currents. Values are mean ± SEM from 3–5 separate oocytes. The IC50 for the αβ-VxXXIVA isomer with disulfide connectivity of I-II; III-IV was 1.49 μM (1.18–1.88) with Hill slope of 0.81 (0.66–0.96). The IC50 for the αβ-VxXXIVA isomer with disulfide connectivity of I-III; II-IV was 3.15 μM (2.08–4.78) with Hill slope of 0.64 (0.46–0.81). Hill slopes (mH) were: Data points shown are the mean ± SEM.
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![Figure 5](image-url)
Figure 6. $^1$H NMR spectra of αB-Conotoxin VxXXIVA isomers. Peptides were dissolved in 20 mM phosphate buffer at pH 5.8 and spectra were acquired at 600 MHz.
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Figure 7. CD spectra αB-Conotoxin VxXXIVA isomers. (A) Overlay of spectra in phosphate buffer. (B) CD spectrum of αB-VxXXIVA [1,2] in the presence of 50 and 87% TFE. This isomer showed a propensity to adopt a partially helical conformation at high TFE concentrations as evident by the shift in the minimum towards 208 nm along with some ellipticity developing around 222 nm.
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peptides has been shown to facilitate oxidative folding [22]. Consequently, the pro region of conotoxins was originally proposed as a means by which these peptides could fold into the same three-dimensional scaffold with identical disulfide connectivity [23]. However, evidence from studies with the two-disulfide \( \alpha \)-conotoxin GI and three disulfide \( \omega \)-conotoxin MVIIA [24] indicates that the propeptide sequence does not necessarily contribute directly to folding thermodynamics but rather plays a facilitative role when folding is catalyzed by a disulfide isomerase [25]. The pro domain has also been implicated in the secretory pathway of hydrophobic O-superfamily conotoxins [26]. Apparently, such a mechanism is not necessary for the more hydrophilic 2B-VxXXIVA.

The mature 2B-VxXXIVA toxin is 40 amino acid residues in length and has a previously unreported arrangement of four Cys residues, C-C-C-C. We synthesized the three possible disulfide isomers (Fig. 2) and assessed their activity at nAChRs. There are no reported examples of conotoxins that contain a vicinal disulfide bridge, and in the present case, the isomer that was synthesized with linkage between the adjacent second and third Cys residues was inactive. Both of the other two possible disulfide connectivities, 2B-VxXXIVA [1,2] with a disulfide connectivity I–II, III–IV, and 2B-VxXXIVA [1,3] with I–III, II–IV, blocked \( \omega \)99\( \omega \)10 nAChRs, with the I–II, III–IV connectivity being 2-fold more active than the I–III, II–IV form.

There is precedent for conotoxins that selectively block the \( \omega \)99\( \omega \)10 over other nAChR subtypes. \( \alpha \)-Conotoxin Vc1.1 from C. victorina and \( \omega \)-conotoxin RgIA from C. regius block the \( \omega \)99\( \omega \)10 nAChR with IC\(_{50}\) values of 5 and 19 nM, respectively [27]. Vc1.1 also blocks \( \alpha \)/\( \omega \)2/\( \omega \)3/\( \omega \)4/\( \omega \)4 nAChRs with IC\(_{50}\) values of 140 and 4200 nM, respectively. Both \( \alpha \)-CTx Vc1.1 and \( \alpha \)-CTx RgIA were subsequently found to activate GABA\(_A\) receptors [27,29,29]. In addition, other conotoxins that block nAChRs have also been reported to block voltage-gated ion channels including sodium and potassium channels [30,31]. The IC\(_{50}\) values for the 2B-VxXXIVA isomers against \( \omega \)99\( \omega \)10 nAChRs are in the micromolar range. It is therefore possible that these peptides, in addition to blocking nAChRs, will subsequently be found to act on other ligand- or voltage-gated ion channels.

Although cone snails hunt fish, molluscs and worms, worms are the most common prey. The nAChR subunits from these polychaete marine worms have not been cloned; however, it is of note that 2B-VxXXIVA preferentially targets the \( \omega \)99\( \omega \)10 subtype of nAChR. The \( \omega \)99 subunit is a member of the nAChR family although it is more distantly related; indeed it appears to be the closest subunit to the ancestor that gave rise to the nAChR family [32]. Thus, it is tempting to speculate that, among Cono, the worm-hunting species may be particularly likely to produce toxins that target \( \omega \)99 receptors.

The \( \omega \)99 subunit is also of increasing interest in biomedicine. Conotoxins that target the \( \omega \)99 nAChR have been shown to be analgesic [10,27] and to accelerate the recovery of function after nerve injury, possibly through immune-mediated mechanisms [33,34]. In addition, small molecule antagonists of \( \omega \)99\( \omega \)10 nAChRs are analgesic in models of neuropathic pain [35,36].

The \( \omega \)99\( \omega \)10 receptor is present in keratinocytes and is implicated in the pathophysiology of wound healing [37]. Recently it has been shown that the \( \omega \)99 subunit is overexpressed in breast cancer tissue. \( \omega \)99 antagonists reduce tumour growth [38,39]. Moreover, variants of the \( \omega \)99 subunit affect transformation and proliferation of bronchial cells [1,40]. Thus, novel antagonists of the \( \omega \)99\( \omega \)10 nAChR are not only of value to structure/function analysis of this receptor subtype but may also help inform development of novel therapeutics.

The 2B-VxXXIVA toxins are atypical among disulfide-bridged conotoxins in showing largely disordered structures in aqueous solution over a range of temperature and pH values. While unusual, this is consistent with structure predictions that show no significant ordered secondary structure for this amino acid sequence (Fig. S3); presumably this is also why the addition of a helix-stabilizing co-solvent like TFE did not induce significant helical structure in 2B-VxXXIVA (Fig. 7). There are, however, precedents for disulfide-bridged conotoxins with poorly ordered structures and potent biological activity. Synthetic \( \alpha \)-AIB, for example, formed both a globular (native) isomer and a ribbon isomer upon oxidative refolding, and the ribbon isomer, although having a less well-defined structure, had approximately 10 times greater potency than the native peptide on nACh-evoked currents in rat parasympathetic neurons [41]. More recently, three different disulfide-bridge isomers of the \( \omega \)-conotoxin PIIIA, which contains three disulfides, were found to block the skeletal muscle voltage-gated sodium channel Na\(_V\)1.4 with similar, yet distinct potencies [42] even though one of them was disordered and gave a poorly dispersed \( \text{^1H} \) NMR spectrum akin to those observed for all three 2B-VxXXIVA disulfide isomers.

The concept of intrinsically disordered proteins is well established now [43], although it is quite unusual to find a conotoxin containing two disulfide bridges that displays these properties, as in the case of 2B-VxXXIVA. It is believed that most intrinsically disordered proteins adopt a more ordered structure upon binding to their physiological targets [44], although evidence is emerging that this is not always the case. It remains to be seen if 2B-VxXXIVA becomes more ordered upon binding to \( \omega \)99\( \omega \)10 nAChR. This might be assessed by studying the interaction of ACh-binding proteins engineered to resemble the \( \omega \)99\( \omega \)10 nAChR [45] and/or by creating conformationally constrained analogues of 2B-VxXXIVA.

Supporting Information

**Figure S1** The amide and aromatic region of \( \text{^1H} \) NMR spectra of 2B-VxXXIVA [1,2] isomer at pH 5.8 and 7.0 in 20 mM phosphate buffer, acquired on a Varian 600 MHz NMR spectrometer at 22 C. Note that fewer amide resonances are observed at pH 7.0 because some are in rapid to intermediate exchange with solvent water at this pH. (TIFF)

**Figure S2** The amide and aromatic region of \( \text{^1H} \) NMR spectra of 2B-VxXXIVA [1,3] isomer at pH 5.8 and 7.0 in 20 mM phosphate buffer, acquired on a Varian 600 MHz NMR spectrometer at 22 C. Note that fewer amide resonances are observed at pH 7.0 because some are in rapid to intermediate exchange with solvent water at this pH. (TIFF)

**Figure S3** The amide and aromatic region of \( \text{^1H} \) NMR spectra of 2B-VxXXIVA [1,4] isomer at pH 5.8 and 7.0 in 20 mM phosphate buffer, acquired on a Varian 600 MHz NMR spectrometer at 22 C. Note that fewer amide resonances are observed at pH 7.0 because some are in rapid to intermediate exchange with solvent water at this pH. (TIFF)

**Figure S4** 1H NMR spectra of 2B-VxXXIVA (Fig. 7). There are, however, precedents for disulfide-bridged conotoxins with poorly ordered structures and potent biological activity. Synthetic \( \alpha \)-AIB, for example, formed both a globular (native) isomer and a ribbon isomer upon oxidative refolding, and the ribbon isomer, although having a less well-defined structure, had approximately 10 times greater potency than the native peptide on nACh-evoked currents in rat parasympathetic neurons [41]. More recently, three different disulfide-bridge isomers of the \( \omega \)-conotoxin PIIIA, which contains three disulfides, were found to block the skeletal muscle voltage-gated sodium channel Na\(_V\)1.4 with similar, yet distinct potencies [42] even though one of them was disordered and gave a poorly dispersed \( \text{^1H} \) NMR spectrum akin to those observed for all three 2B-VxXXIVA disulfide isomers.

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Figure S5 Secondary structure prediction of αB-VxXXIVA isomer, using the PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/).

TIFF

Table S1 Alignment of mature toxin sequences of Conus gene superfamilies from Table 1.

(DOC)

Table S2 Alignment of mature toxin sequences of nAChR targeted conotoxin superfamilies from Table 2.

(DOC)

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(DOC)

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Author Contributions

Conceived and designed the experiments: SL, JMM. Performed the experiments: SL, S. Christensen DZ YW YH XZ S. Chhabra RSN, JMM. Analyzed the data: SL, S. Christensen S. Chhabra RSN JMM. Contributed reagents/materials/analysis tools: S. Christensen YW. Wrote the paper: SL RSN JMM.

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