Backbone cyclization of analgesic conotoxin GeXIVA facilitates direct folding of the ribbon isomer

Xiaosa Wu  
*University of Queensland*

Y H. Huang  
*University of Queensland*

Quentin Kaas  
*University of Queensland*

Peta J. Harvey  
*University of Queensland*

Conan K. Wang  
*University of Queensland*

*See next page for additional authors*

Follow this and additional works at: [https://ro.uow.edu.au/ihmri](https://ro.uow.edu.au/ihmri)

Part of the Medicine and Health Sciences Commons

**Recommended Citation**

Wu, Xiaosa; Huang, Y H.; Kaas, Quentin; Harvey, Peta J.; Wang, Conan K.; Tae, Han Shen; Adams, David J.; and Craik, David J., "Backbone cyclization of analgesic conotoxin GeXIVA facilitates direct folding of the ribbon isomer" (2017). *Illawarra Health and Medical Research Institute*. 1133.  

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au
Backbone cyclization of analgesic conotoxin GeXIVA facilitates direct folding of the ribbon isomer

Abstract
Conotoxin GeXIVA inhibits the α9α10 nicotinic acetylcholine receptor (nAChR) and is analgesic in animal models of pain. α-Conotoxins have four cysteines that can have three possible disulfide connectivities: globular (CysI-CysIII and CysII-CysIV), ribbon (CysI-CysIV and CysII-CysIII), or bead (CysI-CysII and CysIII-CysIV). Native α-conotoxins preferably adopt the globular connectivity, and previous studies of α-conotoxins have focused on the globular isomers as the ribbon and bead isomers typically have lower potency at nAChRs than the globular form. A recent report showed that the bead and ribbon isomers of GeXIVA are more potent than the globular isomer, with low nanomolar half-maximal inhibitory concentrations (IC50). Despite this high potency, the therapeutic potential of GeXIVA is limited, because like most peptides, it is susceptible to proteolytic degradation and is challenging to synthesize in high yield. Here we used backbone cyclization as a strategy to improve the folding yield as well as increase the serum stability of ribbon GeXIVA while preserving activity at the α9α10 nAChR. Specifically, cyclization of ribbon GeXIVA with a two-residue linker maintained the biological activity at the human α9α10 nAChR and improved stability in human serum. Short linkers led to selective formation of the ribbon disulfide isomer without requiring orthogonal protection. Overall, this study highlights the value of backbone cyclization in directing folding, improving yields, and stabilizing conotoxins with therapeutic potential.

Disciplines
Medicine and Health Sciences

Publication Details

Authors
Xiaosa Wu, Y H. Huang, Quentin Kaas, Peta J. Harvey, Conan K. Wang, Han Shen Tae, David J. Adams, and David J. Craik

This journal article is available at Research Online: https://ro.uow.edu.au/ihmri/1133
Backbone cyclization of analgesic conotoxin GeXIVA facilitates direct folding of the ribbon isomer

Received for publication, July 25, 2017 Published, Papers in Press, August 28, 2017, DOI 10.1074/jbc.M117.808386

Xiaosa Wu†, Yen-Hua Huang‡, Quentin Kaas‡, Peta J. Harvey, and David J. Craik‡

From the †Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia and the ‡Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, New South Wales 2522, Australia

Edited by Norma Allweil

Acetylcholine receptors are present in the nervous system and at neuromuscular junctions and are defined as either muscarinic acetylcholine receptors or nicotinic acetylcholine receptors (nAChRs) (1, 2). Neuronal nAChR subtypes are homo- or heteropentamers formed by the combination of α (α2–α10) and β (β2–β4) subunits. These neuronal nAChR subtypes are involved in a range of conditions and diseases, including neuropathic pain, nicotine addiction, schizophrenia, Parkinson’s disease, and Alzheimer’s disease (3, 4).

Conotoxins are disulfide-rich peptides found in the venoms of marine cone snails that can selectively and potently alter the function of ion channels and receptors of the nervous system (5–7). Some have been used as pharmacological probes or developed as drug leads (8, 9). One notable example is ω-conotoxin MVIIA (also called ziconotide or Prialt®), which targets the calcium channel CaV2.2 and is an approved drug to treat neuropathic pain (10, 11). Likewise, Vc1.1 reached phase II clinical trials for the treatment of neuropathic pain, and RgIA also entered phase I trials. These latter two peptides have high potency at the α9α10 nAChR, which is considered a pain target, although the full mechanism(s) of analgesia are not understood (12, 13), and G protein-coupled receptors such as the GABA_B receptor have also been suggested as targets (14–17).

The sequence of conotoxin GeXIVA was identified recently in a transcriptome analysis of the venom duct of Conus generulis (18). This 28-amino acid peptide has four cysteines (Cys I–IV) that can theoretically form three isomers depending on the disulfide connections: globular (CysI–CysIII and CysII–CysIV), ribbon (CysI–CysIV and CysII–CysIII), or bead (CysI–CysII and CysIII–CysIV). Native α-conotoxins preferentially adopt the globular connectivity, and previous studies of α-conotoxins have focused on the globular isoforms as the ribbon and bead isoforms typically have lower potency at nAChRs than the globular form. A recent report showed that the bead and ribbon isoforms of GeXIVA are more potent than the globular isoform, with low nanomolar half-maximal inhibitory concentrations (IC50). Despite this high potency, the therapeutic potential of GeXIVA is limited, because like most peptides, it is susceptible to proteolytic degradation and is challenging to synthesize in high yield. Here we used backbone cyclization as a strategy to improve the folding yield as well as increase the serum stability of ribbon GeXIVA while preserving activity at the α9α10 nAChR. Specifically, cyclization of ribbon GeXIVA with a two-residue linker maintained the biological activity at the human α9α10 nAChR and improved stability in human serum. Short linkers led to selective formation of the ribbon disulfide isomer without requiring orthogonal protection. Overall, this study highlights the value of backbone cyclization in directing folding, improving yields, and stabilizing conotoxins with therapeutic potential.

Conotoxin GeXIVA inhibits the α9α10 nicotinic acetylcholine receptor (nAChR) and is analgesic in animal models of pain. α-Conotoxins have four cysteines that can have three possible disulfide connectivities: globular (CysI–CysIII and CysII–CysIV), ribbon (CysI–CysIV and CysII–CysIII), or bead (CysI–CysII and CysIII–CysIV). Native α-conotoxins preferably adopt the globular connectivity, and previous studies of α-conotoxins have focused on the globular isoforms as the ribbon and bead isoforms typically have lower potency at nAChRs than the globular form. A recent report showed that the bead and ribbon isoforms of GeXIVA are more potent than the globular isoform, with low nanomolar half-maximal inhibitory concentrations (IC50). Despite this high potency, the therapeutic potential of GeXIVA is limited, because like most peptides, it is susceptible to proteolytic degradation and is challenging to synthesize in high yield. Here we used backbone cyclization as a strategy to improve the folding yield as well as increase the serum stability of ribbon GeXIVA while preserving activity at the α9α10 nAChR. Specifically, cyclization of ribbon GeXIVA with a two-residue linker maintained the biological activity at the human α9α10 nAChR and improved stability in human serum. Short linkers led to selective formation of the ribbon disulfide isomer without requiring orthogonal protection. Overall, this study highlights the value of backbone cyclization in directing folding, improving yields, and stabilizing conotoxins with therapeutic potential.

Acetylcholine receptors are present in the nervous system and at neuromuscular junctions and are defined as either muscarinic acetylcholine receptors or nicotinic acetylcholine receptors (nAChRs) (1, 2). Neuronal nAChR subtypes are homo- or heteropentamers formed by the combination of α (α2–α10) and β (β2–β4) subunits. These neuronal nAChR subtypes are involved in a range of conditions and diseases, including neuropathic pain, nicotine addiction, schizophrenia, Parkinson’s disease, and Alzheimer’s disease (3, 4).

Conotoxins are disulfide-rich peptides found in the venoms of marine cone snails that can selectively and potently alter the function of ion channels and receptors of the nervous system (5–7). Some have been used as pharmacological probes or developed as drug leads (8, 9). One notable example is ω-conotoxin MVIIA (also called ziconotide or Prialt®), which targets the calcium channel CaV2.2 and is an approved drug to treat neuropathic pain (10, 11). Likewise, Vc1.1 reached phase II clinical trials for the treatment of neuropathic pain, and RgIA also entered phase I trials. These latter two peptides have high potency at the α9α10 nAChR, which is considered a pain target, although the full mechanism(s) of analgesia are not understood (12, 13), and G protein-coupled receptors such as the GABA_B receptor have also been suggested as targets (14–17).

The sequence of conotoxin GeXIVA was identified recently in a transcriptome analysis of the venom duct of Conus generulis (18). This 28-amino acid peptide has four cysteines (Cys I–IV) that can theoretically form three isomers depending on the disulfide connections: globular (CysI–CysIII and CysII–CysIV), ribbon (CysI–CysIV and CysII–CysIII), or bead (CysI–CysII and CysIII–CysIV) as shown in Fig. 1. Surprisingly, all three isomers of GeXIVA inhibit the rat α9α10 nAChR with similar potency, although the bead and ribbon isoforms are slightly more potent than the globular (IC50 of the bead = 4.6 nm; ribbon = 7.0 nm; and globular = 22.7 nm) (18). NMR structure calculations have shown that ribbon GeXIVA is flexible while still having a more defined structure than the other two isoforms (18). These isoforms exhibit higher or similar potency at rat α9α10 nAChR compared with Vc1.1 (19) or RgIA (20). In addition, the ribbon and bead isoforms of GeXIVA showed promise in relieving neuropathic pain in a chronic constriction injury model and also in a tail-flick model of acute pain (21).

Peptides, including conotoxins, typically have low oral bioavailability and are easily degraded by proteases in vivo, thus decreasing their therapeutic potential (22–24). Over the last decade, cyclic peptides have been discovered in animals, plants, fungi, and bacteria and offer clues toward improving the stability of peptides (25–27). Head-to-tail cyclic peptides are typically more resistant to thermal or enzymatic degradation than their linear counterparts (28, 29). Improving the stability of conotoxins in biological fluids using backbone cyclization was initially inspired by research into naturally occurring cyclic
peptides (29, 30) and is a proven strategy to improve the stability of peptides, including conotoxins (31). For example, the stability of α-conotoxins MII, Iml, Vc1.1, RglA, AudB, and γ-conotoxin MrIA was significantly improved through backbone cyclization, and most of the cyclic peptides had similar or improved activity compared with their parent peptides (32). Most notably, Vc1.1 became orally active in animal models of pain after backbone cyclization (33). The length and amino acid composition of the linker used to join the N and C termini of the parent peptide are vital factors for backbone cyclization. For example, the length of the linker has been shown to modulate the yields of the three isomers of framework I conotoxins (corresponding to a CC-C-C pattern) and influence their biological activities (34–38).

Here we investigated whether a similar strategy could be used for α-conotoxin GeXIVA. We used backbone cyclization to control the selective formation of the ribbon isomer and investigated the effects of cyclization on structure, activity, and stability. The ribbon isomer is formed by the oxidation of two cysteine residues located near the N and C termini of the peptide, suggesting that backbone cyclization could be used to direct the selective formation of the resultant disulfide bond. The three isomers of linear GeXIVA and a suite of cyclic ribbon GeXIVA analogs were synthesized, and their serum stabilities and biological activities at the human α9α10 (hα9α10) nAChR were tested.

**Results**

**Cyclic GeXIVA ribbon isomer retains native structure**

NMR structure calculations for ribbon GeXIVA (18) showed that its N and C termini are maintained in close proximity (~6.4 Å) by a disulfide bridge between the first and last Cys residues (Fig. 1). Five linkers were designed using molecular modeling to bridge the termini, and all were geometrically compatible with the linear peptide structure, with the cyclic backbones maintained within 1 Å root-mean-square deviation (RMSD) of the linear peptide. The data suggested that even a one-residue linker is sufficient to cyclize the backbone of ribbon GeXIVA without perturbing structure. Models of cyclic GeXIVA with minimum perturbation of the parent peptide backbone are shown in Fig. 2. For experimental validation, we focused on three short linkers (G, GG, and GAG) and the three corresponding cyclic variants: ribbon cGeXIVA_G, ribbon cGeXIVA_GG, and ribbon cGeXIVA_GAG. The three isomers of linear GeXIVA and the three cyclic ribbon analogs were synthesized using orthogonal protection of the cysteine side chains to orient the folding toward a unique disulfide isomer. The yields of three isomers of linear GeXIVA and cyclic ribbon GeXIVA were low (<1%) using a two-step oxidation method. Although we optimized the purification procedure, globular GeXIVA could not be completely purified. We therefore explored an alternative one-pot synthetic approach.

**One-pot oxidative folding**

Non-directed oxidative folding of GeXIVA and cGeXIVA_GG was carried out in NH₄HCO₃ (pH 8.3) at room temperature (Fig. 3A). For GeXIVA, three major products were formed within 20 h. The three major peaks were observed in the ultra-performance liquid chromatography trace, accounting for ~25, 45, and 30% of the total peptide, respectively. Analysis of each peak using a TripleTOF 5600 mass spectrometer (AB SCIEX, Concord, Canada) with a nanoelectrospray ionization source revealed masses of 3450.62, 3450.62, and 3450.63 Da, respectively, each corresponding to fully oxidized GeXIVA. The three peaks were unambiguously identified by co-elution with the globular GeXIVA, bead GeXIVA, and ribbon GeXIVA isomers. The HPLC peaks of ribbon and bead GeXIVA partly overlapped and were difficult to separate (Fig. 3A, 24h). Interestingly, a single peak representing the majority of the oxidized forms transiently appeared from 1 to 4 h and then disappeared from 4 to 8 h (Fig. 3A, black asterisk). This transient peak partly overlaps with the major final product (Fig. 3A, 24h), which was identified as globular GeXIVA. We hypothesized that the globular isomer might form within 4 h and then gradually convert into other isomers. We incubated globular GeXIVA in NH₄HCO₃ (pH 8.3) at room temperature at a final concentration of 20 μM and the globular GeXIVA was found to be very stable in the folding buffer for 24 h (data not shown), suggesting that the first major peak at 4 h mainly comprises intermediate forms with similar retention time to the globular isomer. The one-step oxidation of reduced cGeXIVA_GG was complete at 16 h, whereas some intermediates could still be seen at
that time for the linear GeXIVA. Only three products were formed, all of mass 3546.66 Da, corresponding to that of the fully oxidized cGeXIVA GG. The third major peak accounted for 86% of the total peptide content and was confirmed as being the ribbon isomer by co-elution. The two other peaks are presumably the globular and bead isomers. In contrast to linear GeXIVA, the retention time of globular, bead, and ribbon isomers of cGeXIVA GG separate well (Fig. 3A) as the linker engenders ribbon cGeXIVA GG with a larger hydrophobic patch than ribbon GeXIVA (Fig. 3B, green), which could explain its later retention time. Four intermediate forms were identified over the time course. The major intermediate (Fig. 3A, blue asterisk and labeled I1) was the most abundant of the oxidized forms from 1 to 4 h. The major intermediate I1 of cGeXIVA GG was isolated, and its mass was 3548.66 Da, indicating that it has only one disulfide bond. The masses of the three minor intermediates were each 3546.65 Da, suggesting that they have two disulfide bonds (all labeled I2 in Fig. 3A).

NMR analyses of ribbon GeXIVA and ribbon cGeXIVA GG

A comparison of the secondary αH chemical shifts of ribbon GeXIVA with those of ribbon cGeXIVA GG suggested very little difference in structure between the linear and cyclic analogs (Fig. 4A). The different chemical environment created by the presence of the cyclizing linker can easily explain the minimal shift differences near the termini. Overlapping peaks in the 2D NMR spectra prevented the unambiguous assignment of the cGeXIVA GG spin systems.

The 3D structure of cGeXIVA GG was calculated with a total of 171 distance restraints and found to contain a short 310-helix (from Pro12 to Arg16; Fig. 4B). The Ser11–Pro12 peptide bond was determined to adopt a trans configuration, similar to the configuration of this bond in the ribbon and bead isomers of linear GeXIVA (18). As shown for ribbon GeXIVA (18), the loop formed between Cys9 and Cys20 overlays reasonably well between NMR models (RMSD of 1.38 ± 0.56 Å across backbone atoms of residues 9–20; Table 1), although the remainder of the peptide is somewhat disordered, suggesting a degree of flexibility. We thus used NMR spin relaxation measurements to characterize the backbone dynamics of cGeXIVA GG (Fig. 5 and Table 2). Heteronuclear T1 and NOEs were measured and used to derive order parameters reflective of motion of the Ca-Hα bonds. Because of spectral overlap, only 18 of the 30 residues could be analyzed. Nevertheless, the order parameters (0.53–0.81; mean of 0.63) suggest that cGeXIVA GG is moderately flexible, which agrees with the geometric analysis of the lowest energy structures. The least flexible region is at the C-terminal end of the Cys9 to Cys20 loop.

Ribbon cGeXIVA GG and ribbon GeXIVA are equipotent inhibitors of the human α9α10 nAChR subtype

Linear GeXIVA isomers have been shown to selectively and potently inhibit the rat α9α10 nAChR subtype (18). Here, globular, bead, and ribbon isomers of linear GeXIVA, and cyclic constructs of ribbon GeXIVA (cGeXIVA G, cGeXIVA GG, and ribbon cGeXIVA GAG) were tested for their functional activity at the hα9α10 nAChR subtype heterologously expressed in Xenopus laevis oocytes. All three cyclic ribbon constructs tested at 100 nM inhibited ACh-evoked currents mediated by hα9α10 nAChRs by ~60–70% (n = 4–6). Similarly,
Cyclization improves selective folding of conotoxin GeXIVA

A, HPLC traces of samples taken at various times during the random folding process. For GeXIVA, the **black asterisk** indicates that the peak occurring in the folding process of GeXIVA comprises intermediate forms with similar retention time to the globular isomer, and the **red asterisk** indicates an impurity. The globular, bead, and ribbon GeXIVA from two-step oxidations are shown as **red**, **green**, and **blue traces**, respectively. For cGeXIVA_GG, the **blue asterisk** indicates the major intermediate occurring in the folding process of cGeXIVA_GG. The ribbon cGeXIVA_GG from two-step oxidation is shown as a **blue trace**. I₁ represents the intermediate with one disulfide bond, and I₂ represents the intermediates with two disulfide bonds. B, surface representations of ribbon GeXIVA and ribbon cGeXIVA_GG. Positively charged residues are **blue**, negative residues are **red**, polar residues are **white**, and hydrophobic residues are **green**. Surface representations were created using the program PyMOL. Cyclization of ribbon GeXIVA resulted in an additional hydrophobic patch (**circle**) in cGeXIVA_GG.

The concentration-dependent activity of globular and ribbon GeXIVA, and ribbon cGeXIVA_GG at hα9α10 nAChRs were determined (Fig. 6B). All peptides reversibly inhibited ACh-evoked currents in a concentration-dependent manner, giving IC₅₀ values of 198.6 ± 18.9 nm (n = 4 – 12) for globular GeXIVA, 35.1 ± 2.7 nm (n = 4 – 8) for ribbon GeXIVA and 37.6 ± 4.9 nm (n = 4 – 11) for ribbon cGeXIVA_GG. Consistent with the previously reported potency of globular and ribbon GeXIVA at rat α9α10 nAChRs, the globular isomer of GeXIVA inhibited the hα9α10 ACh-evoked current amplitude by ~60–70%. In contrast, the globular GeXIVA was less potent, inhibiting hα9α10 ACh-evoked currents by ~40% (n = 12) (Fig. 6A).

Figure 3. Time course of the oxidative folding of GeXIVA and cGeXIVA_GG. A, HPLC traces of samples taken at various times during the random folding process.
had lower potency at h\textsubscript{10} nAChRs than the ribbon analog. Ribbon GeXIVA and ribbon cGeXIVA\_GG had similar IC\textsubscript{50} values, suggesting that backbone cyclization had no impact on the potency of inhibition at h\textsubscript{10} nAChRs.

Serum stability of linear GeXIVA and ribbon cGeXIVA isomers

Stability assays for the ribbon isomer of GeXIVA and its three cyclic analogs were carried out in human serum. All four peptides degraded within 2 h, but the ribbon GeXIVA\_GG was slightly more stable than the other peptides (data for GeXIVA\_G and GeXIVA\_GAG not shown). Subsequently, the stability of the three isomers of linear GeXIVA and of ribbon cGeXIVA\_GG was compared with that of \textalpha\textsubscript{9}a\textalpha\textsubscript{10} nAChR (33, 47), so studies of new examples such as GeXIVA are important for defining receptor specificity. Furthermore, previous studies have highlighted the value of backbone cyclization to enhance the biopharmaceutical properties of \textalpha\textsubscript{9}a\textalpha\textsubscript{10} nAChRs (33, 47), and so here we attempted to apply this approach to GeXIVA.

Molecular modeling was used to design linkers comprising only a small number of amino acids that would introduce minimal constraints into the peptide fold. A recent study highlighted the importance of the distance between the N and C termini in designing cyclizing linkers (48), but considering distances alone will not address limitations caused by accessible conformations of backbones nor the orientation of the termini. Structural perturbations may be introduced unintentionally into a cyclic peptide when a linker is designed on the basis of distance only, ultimately decreasing biological activity (47, 49). In the case of ribbon GeXIVA, modeling showed that the proximity of the termini would allow cyclization with a single residue linker without affecting the structure. Because non-neutral amino acids in the cyclizing linker sequence can have a negative effect on biological activity (50), only Gly and Ala amino acids were used in the linkers in the current study. These residues are small, flexible, non-charged, and likely to have minimal interference with biological activity. No more than two sequential Gly or Ala were used in any individual linker to avoid overlapping peaks in NMR spectra and thus avoid spectral assignment.

Table 1

<table>
<thead>
<tr>
<th>Statistical analysis of cGeXIVA_GG structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>All statistics are given as means ± S.D.</td>
</tr>
<tr>
<td>Experimental restraints</td>
</tr>
<tr>
<td>Total no. distance restraints</td>
</tr>
<tr>
<td>Intraresidue</td>
</tr>
<tr>
<td>Sequential</td>
</tr>
<tr>
<td>Medium range, i − j &lt; 5</td>
</tr>
<tr>
<td>Long range, i − j ≥ 5</td>
</tr>
<tr>
<td>Hydrogen bond restraints</td>
</tr>
<tr>
<td>Dihedral angle restraints</td>
</tr>
<tr>
<td>NOE violations exceeding 0.3 Å</td>
</tr>
<tr>
<td>Dihedral violations exceeding 2.0 Å</td>
</tr>
<tr>
<td>RMSD from mean structure, Å</td>
</tr>
<tr>
<td>Backbone atoms (residues 9–20)</td>
</tr>
<tr>
<td>All heavy atoms (residues 9–20)</td>
</tr>
<tr>
<td>Stereochemical quality\textsuperscript{a}</td>
</tr>
<tr>
<td>Residues in most favored Ramachandran region, %</td>
</tr>
<tr>
<td>Ramachandran outliers, %</td>
</tr>
<tr>
<td>Unfavorable side-chain rotamers, %</td>
</tr>
<tr>
<td>Clashscore, all atoms</td>
</tr>
<tr>
<td>Overall MolProbity score</td>
</tr>
</tbody>
</table>

\textsuperscript{a} According to MolProbity (76).
difficulties. Linkers composed of Gly and Ala residues have been successfully used to cyclize the \( \text{H} \), -conotoxins MII, RgIA, and Vc1.1, without adversely affecting biological activity or structure (33, 47, 49).

The linkers used in this study did not affect the activity and structure of ribbon GeXIVA. Indeed, all cyclic ribbon variants, i.e. cGeXIVA_G, cGeXIVA_GG, and cGeXIVA_GAG, had similar activity to the linear ribbon form at the \( \text{H} \). All cyclic variants, i.e. cGeXIVA_G, cGeXIVA_GG, and cGeXIVA_GAG, had similar activity to the linear ribbon form at the \( \text{H} \). The non-directed oxidation of GeXIVA resulted in the preferential formation of the bead isomer, followed by the ribbon and globular forms, which interestingly parallels their biological activity (18).

The non-directed oxidation of GeXIVA resulted in the preferential formation of the bead isomer, followed by the ribbon and globular forms, which interestingly parallels their biological activity (18). By contrast, two previous studies employing the one-step oxidation of \( \alpha \)-conotoxins showed preferential...
formation of the globular isomer under similar conditions (0.1 M NH₄HCO₃, pH 8, room temperature) (52, 53). A possible explanation for this difference in isomer formation is that GeXIVA displays a Cys framework XIV (C-C-C-C) with 6, 10, and 6 residues within the CysI–CysII, CysII–CysIII, and CysIII–CysIV backbone segments, respectively, rather than the Cys framework I (CC-C-C) of most /H9251-conotoxins with 0, 4, and 3–7 residues between the cysteines.

The one-step oxidation of cGeXIVA_GG was notably different from that of the linear parent peptide because it resulted in the preferential formation of the ribbon isomer. The use of short cyclizing linkers has also been reported to drive oxidative folding toward the ribbon connectivity for three other conotoxins: /H9251-conotoxins ImI and AuIB and /H9273-conotoxin MrIA. Like GeXIVA, these peptides have four Cys residues, with CysI and CysIV being no more than three residues from the N and C termini, respectively. A short cyclizing linker should maintain these two Cys residues in spatial proximity in the reduced form and favor their cross-linking by a disulfide bond during oxidation.

During the oxidative folding of ribbon GeXIVA and ribbon cGeXIVA_GG, a major intermediate was identified. For GeXIVA, the amount of ribbon and globular isomers plateaued after 8 and 12 h of incubation, respectively, whereas the bead form plateaued after 20 h. According to Wedemeyer et al. (54), the rate of formation of disulfide bonds in an unstructured chain decreases with the number of residues between the two cysteines. Because 6, 10, and 6 residues exist between successive cysteines in GeXIVA, we speculate that the major intermediate might involve a disulfide bond between CysI and CysII or between CysIII and CysIV and that this intermediate subsequently translates into the bead isomer. For cGeXIVA_GG, there are only four residues between CysI and CysIV after backbone cyclization, which may favor the formation of either a CysI–CysIV or CysII–CysIII disulfide bond in the intermediate, thus leading to the ribbon isomer. Interestingly, three intermediates with two disulfide connectivities appeared during the course of folding of cGeXIVA_GG. The retention times of these three intermediates were different from the three stable isomers, suggesting that the intermediates have native-like connectivities but have meta-stable conformations.

We hypothesize that the preferential formation of the ribbon isomer probably results from the destabilization of the alternative connectivities rather than through stabilization of the ribbon form (Fig. 8). Backbone cyclization of ribbon GeXIVA possibly affects the folding energy through an entropic effect, mainly impacting the unfolded state. Indeed, the linker has little interaction with the rest of the peptide in molecular models, suggesting that the termini are already maintained in proximity in the linear form, and cyclization does not result in structural perturbation to the core of the peptide. By contrast, the bead and globular isomers probably adopt a different set of conformations after being backbone cyclized because NMR characterization of these isomers indicates that the globular form is disordered, and the N and C termini of the bead isomer are uncoupled (18).

A two-step oxidation strategy is often used to oxidize conotoxins containing two disulfide bonds because the yields are
Cyclization improves selective folding of conotoxin GeXIVA

Typically improved compared with undirected folding (55). However, the oxidation of GeXIVA using this strategy only produced small amounts of products, hindering high-throughput structure-activity relationship studies and therapeutic studies. The yield of folded ribbon GeXIVA calculated from the final weight of oxidized peptide divided by the weight of reduced peptide was 0.5%. Using the results of Cheneval et al. (56) to extrapolate the yield for synthesis of an unprotected peptide, the yield of cyclic ribbon cGeXIVA_GG through a one-step folding method should be 8.6% using Fmoc-based solid-phase peptide synthesis and considerably higher for a Boc-based approach. Additionally, the HPLC elution peaks of ribbon and bead isomers of GeXIVA overlapped, whereas the ribbon isomer of cGeXIVA_GG is distinct, and ribbon cGeXIVA_GG therefore can be produced in greater purity using one-step oxidation than the ribbon GeXIVA using the same strategy.

An alternative strategy to increase the oxidative folding yield has been used in other cases of disulfide-rich peptides, including the widely used model system bovine pancreatic trypsin inhibitor (BPTI). Undirected folding of this protein results in the formation of two main species, one being native BPTI and the other a native-like two-disulfide intermediate (57). The addition of the N-terminal pro-region increased substantially the native-like structure (66) but with an attendant loss of biological activity (67). In a later study, Hua et al. (68) used a six-residue linker, and the resulting variant maintained biological activity and structure and also had improved folding yield and stability. More recently, the nature of the linker region between the two insulin chains was further investigated, resulting in the identification of an insulin variant with the highest folding efficiency to date (64). In that study, it was shown that the linker region had a direct effect on folding yield by enhancing the formation of the native disulfide bond connectivity. The peptides in the current study have a direct analogy to single-chain insulin analogs: a suitable linker for cGeXIVA_GG led to preferential formation of the ribbon isomer and significantly improved the product yield and stability while retaining the native-like structure and biological activity. The enhanced pharmaceutical properties deriving from a suitable linker provide an opportunity to develop GeXIVA as a potential therapeutic for the treatment of neuropathic pain, a condition with a huge unmet medical need (69). Treatment of neuropathic pain is challenging, and alternative therapeutics are needed because currently available drugs such as morphine, gabapentin, or antidepressants are subject either to the development of tolerance or to significant side effects (70, 71).

In summary, the head-to-tail backbone cyclization via a short linker of ribbon GeXIVA had no impact on the potency of inhibition at hα9α10 nAChR and a rationally designed linker favored the formation of the ribbon disulfide connectivity and improved stability in human serum. The high-yield production of ribbon cGeXIVA_GG compared with linear GeXIVA should facilitate future in vivo studies of the analgesic potential of this compound.

Experimental procedures

Design of linkers

Linkers were designed using molecular modeling. The structure of the ribbon isomer cyclized with linkers from one to five amino acid residues was modeled. Fifty models of the backbone cyclized ribbon GeXIVA were generated for each of the linkers using Modeler 6v13 (72), and the conservation of ribbon GeXIVA native conformation was measured by computing the
mean Ca root-mean-square deviation between the models and the 20 lowest-energy NMR structures (18).

**Peptide synthesis, cyclization, oxidation, and purification of GeXIVA and variants**

The three isomers of GeXIVA and the three cyclic analogs (cGeXIVA_G, cGeXIVA_GG and cGeXIVA_GAG) were synthesized using Fmoc-based solid-phase peptide synthesis on an acid-sensitive resin 2-chlorotrityl chloride. Side chains of Cys residues were protected in pairs with orthogonal protective groups that can be removed selectively under different oxidation conditions. The acid-labile trityl group was used to protect the side chains of Cys2 and Cys27, and the acetamidomethyl group was used to protect Cys9 and Cys20 for the ribbon GeXIVA and its cyclic variants. The backbone cyclization of cyclic variants was achieved using the protocol described recently (56). Briefly, a solution of 1% TFA in dichloromethane (v/v) was used to cleave the linear precursor peptides of the cyclic variants off the resin. The side chain protected precursor peptides were dried, cyclized in O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (5 mM) and N,N′-dissopropylethylamine (10 mM) in N,N-dimethylformamide for 3 h and lyophilized. The protecting group on side chains was removed in TFA with 2% triisopropylsilane and 2% water (v/v) for 2 h. The three isomers of GeXIVA were cleaved and deprotected in TFA with 2% triisopropylsilane and 2% water (v/v) for 2 h.

Two-step oxidative folding was used to form disulfide bonds: the first disulfide bond was formed in 0.1 M ammonium bicarbonate (NH4HCO3, pH 8.3) at room temperature (21–24 °C) for 24 h, and the second disulfide bond was formed in 0.1 M iodine solution for 1 h. All peptides were purified by reversed-phase (RP)-HPLC using Phenomenex C18 columns, and the purification method is 15–25% buffer B within 40 min (increasing 1% buffer B per 4 min) in an analytical Vydac C18 column.

**NMR spectroscopy**

Samples of ribbon GeXIVA and its cyclic analogs were prepared by dissolving peptide (~1 mg) in 90% H2O, 10% D2O at a pH of ~3. The spectra were recorded on a Bruker Avance III 600 MHz NMR spectrometer at 298 K and included total correlation spectroscopy (mixing time of 80 ms), NOESY (mixing time of 200 ms), and H-N and H-C heteronuclear single quantum coherence. Chemical shifts were referenced to 600 MHz NMR spectrometer at 298 K and included total correlation spectroscopy (mixing time of 80 ms), NOESY (mixing time of 200 ms), and H-N and H-C heteronuclear single quantum coherence. Chemical shifts were referenced to 5m M HEPES, pH 7.4.

**Electrophysiological recordings**

Electrophysiological recordings were carried out 2–5 days after cRNA microinjection. The oocytes expressing ha9α10...
nAChRs were incubated in 100 μM BAPTA-AM ~3 h before recording. Two-electrode voltage clamp recordings were performed at room temperature using a GeneClamp 500B amplifier and pClamp9 software interface (Molecular Devices, Sunnyvale, CA) at a holding potential ~80 mV. Voltage-recording and current-injecting electrodes were pulled from GC150T-7.5 borosilicate glass (Harvard Apparatus, Holliston, MA) and filled with 3 M KCl, giving resistances of 0.3–1 MΩ. The oocytes were perfused with ND115 solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, and 10 mM HEPES, pH 7.4, using a continuous Legato 270 push/pull syringe pump perfusion system (KD Scientific, Holliston, MA) at a rate of 2 ml/min.

Initially, the oocytes were briefly washed with ND115 solution followed by three applications of ACh at half-maximal effective concentration (EC50) of 6 μM ACh for h9a9α10 nAChR. Washout with bath solution was done for 3 min between ACh applications. The oocytes were incubated with peptides for 5 min with the perfusion system turned off, followed by co-application of ACh and peptide with flowing bath solution. All peptide solutions were prepared in ND115 + 0.1% bovine serum albumin. Peak current amplitudes before (ACh alone) and after (ACh + peptide) peptide incubation were measured using Clampfit 10.7 software (Molecular Devices), where the ratio of ACh + peptide-evoked current amplitude to ACh-alone-evoked current amplitude was used to assess the activity of the peptides at human nAChRs.

All electrophysiological data were pooled (n = 4 to 12) and represent the means ± S.E. Concentration–response curves were obtained using GraphPad Prism 7 nonlinear regression analysis (GraphPad Software, La Jolla, CA). The half-maximal inhibitory concentration (IC50) was determined from the concentration–response curve and reported with error of the fit.

Stability assays

Serum stability of peptides were carried out in 25% human serum (human male AB plasma; Sigma-Aldrich) over 4 h using a 20 μM final peptide concentration. Serum was prepared by centrifugation at 17,000 × g for 15 min to remove the lipid component, and the supernatant was diluted in 1 in 4 phosphate-buffered saline and incubated at 37 °C for 15 min before the assay. Each peptide was incubated in 25% human serum at 37 °C, and aliquots were taken out at time points 0, 1, 2, 3, and 4 h, respectively. Serum proteins were denatured with 2 M urea and precipitated with 20% TFA. All samples were centrifuged at 17,000 × g for 10 min, and the supernatant was analyzed by analytical RP-HPLC. Remaining peptides were quantified using peak height with absorption at 214 nm.


Cyclization improves selective folding of conotoxin GeXIVA

Acknowledgments—We thank Alun Jones and Eivind Undheim (Institute for Molecular Bioscience, The University of Queensland) for mass spectrometry technical support.

References

Cyclization improves selective folding of conotoxin GeXIVA


Cyclization improves selective folding of conotoxin GeXIVA


Backbone cyclization of analgesic conotoxin GeXIVA facilitates direct folding of the ribbon isomer
Xiaosa Wu, Yen-Hua Huang, Quentin Kaas, Peta J. Harvey, Conan K. Wang, Han-Shen Tae, David J. Adams and David J. Craik

doi: 10.1074/jbc.M117.808386 originally published online August 28, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.808386

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 75 references, 11 of which can be accessed free at http://www.jbc.org/content/292/41/17101.full.html#ref-list-1