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α O-Conotoxin GeXIVA Disulfide Bond Isomers Exhibit Differential Sensitivity for Various Nicotinic Acetylcholine Receptors but Retain Potency and Selectivity for the Human $\alpha 9\alpha 10$ Subtype

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Abstract

Nicotinic acetylcholine receptor (nAChR) subtypes exhibit distinct neuropharmacological properties that are involved in a range of neuropathological conditions, including pain, addiction, epilepsy, autism, schizophrenia, Tourette's syndrome, Alzheimer's and Parkinson's diseases, as well as many types of cancer. The $\alpha 9\alpha 10$ nAChR is a potential target in chronic pain, wound healing, the pathophysiology of the auditory system, and breast and lung cancers. α O-conotoxin GeXIVA is a potent antagonist of rat $\alpha 9\alpha 10$ nAChRs, with the 'bead' disulfide bond isomer displaying the lowest IC₅₀ of the three possible isomers. In the rat chronic constriction injury model of neuropathic pain, this isomer reduced mechanical hyperalgesia without affecting motor performance. Here, we report the effects of the three disulfide bond isomers of GeXIVA on human $\alpha 9\alpha 10$ nAChRs, other human nAChR subtypes, various rat nAChR subtypes, and 10 rat $\alpha 9\alpha 10$ nAChR mutants. The three isomers displayed only ~5-fold difference in potency on the human vs rat $\alpha 9\alpha 10$ receptors and had similar affinities at wild-type rat $\alpha 9\alpha 10$ nAChRs and all 10 $\alpha 9\alpha 10$ receptor mutants. From these findings, the binding site and mechanism of action of GeXIVA on rat and human $\alpha 9\alpha 10$ nAChR was deduced to be different from that of other conotoxins targeting this nAChR subtype. GeXIVA is therefore a unique ligand that might prove useful for further probing of binding sites on the $\alpha 9\alpha 10$ nAChR.

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Conflict of interest

The sequence of GeXIVA is patented by Hainan University, with Sulan Luo, Dongting Zhangsun, Yong Wu, Xiaopeng Zhu, Yuanyan Hu and J. Michael McIntosh listed as inventors.

Keywords

α O-conotoxin GeXIVA disulfide bond Isomers; human and rat nAChRs; α 9 α 10 nAChR subtype and its mutants; Differential sensitivity; Mechanism of action

1. Introduction

For centuries, the shells of *Conus* snails have been coveted and collected owing to their beauty, but with this beauty comes danger; cone snails are venomous, immobilizing their prey using a complex concoction of toxins. Each concoction contains a unique complement of a thousand or more diverse components (Davis et al., 2009; Lavergne et al., 2015). Given the existence of more than 800 species of cone snails (Puillandre et al., 2015; Uribe et al., 2017) (REF), the library of toxins is immense. A number of cases of human fatalities from cone snail envenomation have occurred, and reports of “painless death” sparked interest in the analgesic properties of the venom (Livett et al., 2006). Many of the venom compounds, which are small disulfide-rich peptides called conotoxins, have been developed into subtype-selective probes for receptors and ion channels. The sequences of conotoxins are compiled in an online database, ConoServer (Kaas et al., 2012), and their classification schemes and pharmacological activities have been extensively reviewed (Halai and Craik, 2009).

Species differences in target receptors have played a critical role in the development of conotoxins as potential therapeutic agents. One venom component, ω -conotoxin MVIIA, paralyzes fish by targeting calcium channels at the neuromuscular junction. Although calcium channels at the human neuromuscular junction are resistant to ω -conotoxin MVIIA, N-type calcium channels in the dorsal horn of the human spinal cord are highly sensitive to the same toxin. This discovery ultimately led to FDA approval of a chemically synthesized form of ω -conotoxin MVIIA (ziconotide) for use in intractable pain. Another conotoxin, Vc1.1, a potent antagonist of rat α 9 α 10 (α 9 α 10) nicotinic acetylcholine receptors (nAChRs) and an analgesic in rat models of neuropathic pain (Satkunanathan et al., 2005; Vincler et al., 2006) reached phase 2 human clinical trials. However, the discovery that its potency at human α 9 α 10 nAChRs *in vitro* was significantly less than in rat, prompted discontinuation of its clinical development.

A structurally novel 28-amino acid peptide, α O-conotoxin (α O-CTx) GeXIVA, which potently blocks nAChRs, was recently discovered in the transcriptome of *Conus generalis* (Luo et al., 2015). nAChRs are ligand-gated ion channels composed of five subunits, which are either all α subunits or a combination of α and non- α subunits. In mammals, there are nine different α subunits: α 1– α 7, α 9, α 10, four β subunits and γ , δ and ϵ subunits. GeXIVA displays high potency for α 9 α 10 nAChRs. GeXIVA has four cysteine (Cys) residues in its primary sequence, which can be oxidized to form three possible disulfide isomers, referred to as the globular, ribbon and bead isomers. For convenience we hereafter denote them as GeXIVA[1,3], GeXIVA[1,4], and GeXIVA[1,2], respectively, with the numerals in square brackets referring to the first disulfide connectivity in each of the isomers (with the 2nd disulfide connectivity following by default). Remarkably, each of these isomers shows antagonist activity at rat α 9 α 10 nAChRs (Luo et al., 2015). Here we

investigate the subtype-selectivity of each of the isomers on a range of rat nAChRs and at some human nAChR subtypes. We also probe the potential binding site of the peptide using 10 rat $\alpha 9\alpha 10$ receptor mutants.

2. Materials and Methods

2.1. Peptide synthesis

The three isomers of αO -CTx GeXIVA were synthesized as previously described, whereby we used directed synthesis in a two-step oxidation procedure to enforce the disulfide arrangement (Luo et al., 2015). Briefly, the linear peptide was assembled using solid-phase methodology on an ABI 433A peptide synthesizer using FastMoc (N-(9-fluorenyl) methoxycarbonyl) chemistry and standard side-chain protection, except for Cys residues. The Cys residues of the three isomers were protected in pairs with either S-trityl on Cys20 and Cys27 (designated GeXIVA[1,2]), Cys9 and Cys27 (designated GeXIVA[1,3]), Cys9 and Cys20 (designated GeXIVA[1,4]), or S-acetamidomethyl on Cys2 and Cys9, Cys2 and Cys20, Cys2 and Cys27. The peptides were released from the solid support by treatment with reagent K (trifluoroacetic acid/water/ethanedithiol/phenol/thioanisole; 90:5:2.5:7.5:5, v/v/v/v/v). The released peptides were precipitated, washed three times with cold ether, and subjected to a two-step oxidation protocol to fold them selectively (Dowell et al., 2003; Walker et al., 1999). The oxidised peptides were purified using high-performance liquid chromatography on a reversed-phase C18 Vydac column using a linear gradient of 10–50% B60 in 40 min. Solvent B (60% ACN, 40 % H₂O, 0.092% TFA); Solvent A (0.1% trifluoroacetic acid in H₂O). Peptide concentration was measured using a spectrophotometer at a wavelength of 280 nm, and calculated using the Beer-Lambert equation. Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry and NMR spectroscopy were used to confirm the identity of the three disulfide isomers, as described in our earlier study (Luo et al., 2015).

2.2. nAChR clones and cRNA preparation

Clones of rat $\alpha 2$ – $\alpha 7$ and $\beta 2$ – $\beta 4$, as well as mouse muscle $\alpha 1\beta 1\delta\epsilon$ cDNAs, were kindly provided by S. Heinemann (Salk Institute, San Diego, CA). Clones for rat $\alpha 9$ and $\alpha 10$ were kindly provided by A. B. Elgoyhen (INGEBI, Buenos Aires, Argentina). Clones of $\beta 2$ and $\beta 3$ subunits in the high-expressing pGEMHE vector were kindly provided by C.W. Luetje (University of Miami, Miami, FL). Clones of human $\alpha 1\beta 1\delta\epsilon$ were from Steven Sine (Mayo Clinic, Rochester, Minn). The human $\alpha 6$ clone was from Jon Lindstrom (Perelman School of Medicine, Philadelphia, PA). The other human subunits were from James Garrett (Formerly of Cognetix Inc., Salt Lake City, Utah). Clones for the human $\alpha 9$ and $\alpha 10$ subunits were kindly provided by Lawrence Lustig (Johns Hopkins University, Baltimore, MD). The human subunits were subcloned into the pSGEM vector for *Xenopus* oocyte expression. Capped cRNA for the various subunits were made using the mMessage mMachine *in vitro* transcription kit following linearization of the plasmid. The cRNA was purified using the Qiagen RNeasy kit. The concentration of cRNA was determined by absorbance at 260 nm.

2.3. Construction of rat $\alpha 9\alpha 10$ nAChR mutants

Rat $\alpha 9$ or $\alpha 10$ nAChR subunit clones were mutated using the QuikChange system (Stratagene) according to amino acid sequence alignment of N-terminal-binding regions of rat and human $\alpha 9$ and $\alpha 10$ nAChR subunits. In mutated ra9A24K $\alpha 10$ nAChRs, the codon for Ala24 of the rat $\alpha 9$ subunit was replaced by a codon for Lys, and in ra9 $\alpha 10$ E61S nAChRs, the codon for Glu of the rat $\alpha 10$ subunit was replaced by a codon for Ser, and so on, as described previously (Azam and McIntosh, 2012). Briefly, primers containing the desired point mutation flanked by at least 15 bases on either side were synthesized. Using the non-strand displacing action of Pfu Turbo DNA polymerase, the mutagenic primers were extended and incorporated by PCR. The methylated, non-mutated parental cDNA was digested with *DpnI*. The mutated DNA was transformed into DH10B or DH5 α competent cells, isolated using the Qiaprep mini prep kit (Qiagen, Valencia, CA, USA), and sequenced to ascertain the incorporation of the desired mutation.

2.4. cRNA injection and expression

Xenopus oocytes were used to heterologously express cloned nAChR subtypes. Oocyte preparation, and expression of nAChR subunits in *Xenopus* oocytes were performed as described previously (McIntosh et al., 2005). For rat $\alpha 9$ subunit mutants, cRNA of mutant $\alpha 9$ subunit were mixed at a 1:1 ratio with wild-type $\alpha 10$ for a final concentration of at least 500 ng/ μ L for each subunit cRNA. For rat $\alpha 10$ subunit mutants, cRNA of mutant $\alpha 10$ subunit were mixed at a 1:1 ratio with wild-type $\alpha 9$. Fifty to 150 nL of this mixture was injected into each *Xenopus* oocyte using a Drummond microdispenser (Drummond Scientific, Broomall, PA), as described previously (Cartier et al., 1996), which were incubated at 17°C. Oocytes were injected within a day of harvesting and recordings were made 2–5 days post-injection.

2.5. Electrophysiology

Oocytes were voltage-clamped and exposed to acetylcholine (ACh) and peptide, as described previously (Cartier et al., 1996). The oocyte recording chamber was fabricated from Sylgard and was 30 μ L in volume. All recordings were done at room temperature (~22°C). Briefly, oocytes were gravity-perfused with ND-96 buffer containing 1 μ M atropine and 0.1 mg/ml BSA at a rate of ~2 mL/min. The ND-96 buffer, with a pH of 7.1–7.5, consisted of 96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂ and 5 mM HEPES. In the case of rat and human $\alpha 9\alpha 10$, $\alpha 7$, and mouse muscle $\alpha 1\beta 1\delta\epsilon$ subtypes, to avoid antagonism of $\alpha 7$ -like receptors atropine was omitted from the buffer (Cartier et al., 1996). ACh-gated currents were obtained using a two-electrode voltage-clamp amplifier (model OC-725B, Warner Instruments, Hamden, CT). The membrane potential was clamped at –70 mV for all nAChR subtypes and mutants. The perfused oocyte was then subjected to a 1-sec pulse of 100 μ M ACh once a minute repeatedly. In the case of rat and human $\alpha 9\alpha 10$ and mouse/human muscle $\alpha 1\beta 1\delta\epsilon$ subtypes a 1-sec pulse of 10 μ M ACh was used, and for the rat and human $\alpha 7$ subtype a 1-sec pulse of 200 μ M ACh was used. To measure conotoxin antagonism, peptide was bath-applied for 5 min before exposure to ACh. All ACh pulses contained no toxin.

2.6. Statistical Analysis

The IC₅₀ values for the oocyte-expressing receptors are reported with 95% confidence intervals. The average of five control responses just preceding a test response was used to normalize the test response to obtain “percent response.” IC₅₀ values were determined by nonlinear regression analysis using Prism (GraphPad Software, Inc., San Diego, CA, USA). All statistical analyses were performed with Prism. Each data point of a concentration-response curve represents the average $\pm$ S.E.M. of at least three oocytes.

2.7. Molecular modelling

The molecular models of the two binding modes of ribbon GeXIVA on the $\alpha 9\alpha 10$ nAChR that we proposed previously (Luo et al., 2015) were mutated using Modeller9v13 (Sali and Blundell, 1993) for use in the current study. The mutated models were minimised and submitted to 5 ns molecular dynamics simulations using Gromacs 2016 (Abraham et al., 2015) and the Amber99sb-ILDN force field (Lindorff-Larsen et al., 2010). Buried surface areas were computed using the Gromacs package.

3. Results

3.1. GeXIVA isomers are selective for $\alpha 9\alpha 10$ nAChRs

The three disulfide isomers of GeXIVA were tested for their potency against the rat $\alpha 9\alpha 10$ ($\alpha 9\alpha 10$) nAChR, as described previously (Luo et al., 2015). We further investigated the effect of the three isomers on other subunit combinations of neuronal and muscle nAChRs heterologously expressed in *Xenopus* oocytes (Table 1, Fig. 1). Concentration-response analysis indicated that, unlike most α -conotoxins, all three isomers displayed potency against a broad spectrum of rat nAChR subtypes. The three isomers most potently blocked the $\alpha 9\alpha 10$ nAChRs. Generally, the three isomers displayed the lowest potency for subtypes containing the $\beta 4$ nAChR subunit, and had little effect on $\alpha 2\beta 4$, $\alpha 3\beta 4$ and $\alpha 4\beta 4$ nAChRs, with IC₅₀s > 869 nM.

Despite their potency, the three isomers exhibited differential sensitivity to rat nAChR subtypes. All three isomers were highly selective for $\alpha 9\alpha 10$ nAChR, with IC₅₀s ranging from 4.6–22.7 nM. All three isomers were less potent on the rat $\alpha 7$ nAChR subtype. Of the isomers, GeXIVA[1,2] was most potent at the $\alpha 7$ nAChR subtype, with an IC₅₀ of 432 nM, which is 10-fold more potent than GeXIVA[1,3] (IC₅₀: 4990 nM), and 4.1-fold more than GeXIVA[1,4] (IC₅₀: 1768 nM). Unlike the $\alpha 9\alpha 10$ and $\alpha 7$ subtypes, the three isomers blocked the mouse muscle nAChR subtype ($\alpha 1\beta 1\delta\epsilon$) and the $\alpha 6/\alpha 3\beta 2\beta 3$ subtype with similar potency: IC₅₀s at these subtypes ranged from 394 to 683 nM and 369 to 555 nM, respectively (Table 1, Fig. 1). The three isomers had similar affinities for the $\alpha 3\beta 2$ subtype, with IC₅₀s ranging from 480 to 613 nM (GeXIVA[1,2] and GeXIVA[1,4] both displayed a similar IC₅₀ of 480 nM and 489 nM) (Table 1, Fig. 1). From these findings, we conclude that there are no differences between the three isomers in terms of potency and selectivity for the $\alpha 1\beta 1\delta\epsilon$, $\alpha 6/\alpha 3\beta 2\beta 3$, and $\alpha 3\beta 2$ subtypes, but overall, their potency is far less at these receptors than at the rat and human $\alpha 9\alpha 10$ nAChRs. Thus, GeXIVA[1,3] and GeXIVA[1,4] are slightly less selective for $\alpha 9\alpha 10$ nAChR than GeXIVA[1,2], but are 15-fold more selective for $\alpha 9\alpha 10$ nAChR than the other aforementioned subtypes.

The order of potency of the isomers at the rat $\alpha 9\alpha 10$ subtype differed to that observed at the $\alpha 2\beta 2$, $\alpha 4\beta 2$, $\alpha 2\beta 4$, $\alpha 4\beta 4$, $\alpha 6/\alpha 3\beta 4$ and $\alpha 3\beta 4$ subtypes (Table 3, Fig. 3). GeXIVA[1,4] displayed the greatest potency at $\alpha 2\beta 2$ and $\alpha 4\beta 2$, with IC_{50} s of 122 nM at $\alpha 2\beta 2$, and an IC_{50} of 215 nM at $\alpha 4\beta 2$. The IC_{50} s of GeXIVA[1,2] and GeXIVA[1,3] at $\alpha 2\beta 2$ were 499 nM and 348 nM, respectively, and 1350 nM and 1261 nM, respectively, at $\alpha 4\beta 2$. GeXIVA[1,4] and GeXIVA[1,3] showed similar activities at $\alpha 2\beta 4$, $\alpha 4\beta 4$ and $\alpha 6/\alpha 3\beta 4$. For these six subtypes, GeXIVA[1,2] was the least active of the three isomers, which is interesting given that it had the highest selectivity and was at least 85-fold more potent at $\alpha 9\alpha 10$ than the other subtypes.

3.2. GeXIVA isomers retain potency for $\alpha 9\alpha 10$ nAChRs

The three GeXIVA isomers were tested for their ability to antagonize the response elicited by ACh on the human $\alpha 9\alpha 10$ ($\alpha 9\alpha 10$) nAChR, and four closely related human subtypes (Table 2). The three isomers of GeXIVA block human $\alpha 9\alpha 10$ nAChRs differently. Fig. 2 shows representative responses of $\alpha 9\alpha 10$ nAChRs to ACh in the presence and absence of the three GeXIVA isomers. The block of $\alpha 9\alpha 10$ nAChR induced by both GeXIVA[1,2] and GeXIVA[1,3] was rapidly reversible (Fig. 2A–B), whereas that induced by GeXIVA[1,4] was slower to reverse (Fig. 2C). The most potent isomer was found to be GeXIVA[1,2], which blocked $\alpha 9\alpha 10$ nAChRs almost completely at 100 nM (Fig. 2A). GeXIVA[1,3] was the least potent with a 57% response at 100 nM (Fig. 2B). GeXIVA[1,4] fell in the middle, with a 33% response at 100 nM (Fig. 2C). Concentration–response experiments were conducted (Table 2, Fig. 3), and the IC_{50} of the most potent isomer, GeXIVA[1,2], was observed to be 20.3 (12.4–33.2) nM and the IC_{50} of the least potent isomer, GeXIVA[1,3], was 116 (65.4–204) nM. The IC_{50} of GeXIVA[1,4] fell in-between, at 47.3 (29.7–75.3) nM – 2.3-fold less potent than GeXIVA[1,2] and 5.7-fold more potent than GeXIVA[1,3].

3.3. GeXIVA isomers are selective for $\alpha 9\alpha 10$ nAChR

The concentration–response for the three isomers was assessed against each of the other closely related human nAChR subtypes (see Table 2, Fig. 3). The most potent isomer at $\alpha 7$ nAChR was GeXIVA[1,2], with an IC_{50} of 559 nM, and the least potent was GeXIVA[1,3] with an IC_{50} of 3584 nM. GeXIVA[1,4] again fell in-between with an IC_{50} of 889 nM – 1.6-fold less potent than GeXIVA[1,2] and 4.0-fold more potent than GeXIVA[1,3]. The order of potency of the isomers ([1,2] > [1,4] > [1,3]) was again the same for both the $\alpha 7$ and $\alpha 9\alpha 10$ nAChRs. Unlike the $\alpha 9\alpha 10$ and $\alpha 7$ subtypes, the three isomers were equally potent against human muscle nAChR subtype ($\alpha 1\beta 1\delta\epsilon$) with IC_{50} s of 497 nM ([1,2]), 485 nM ([1,3]), and 365 nM ([1,4]), < 1.4-fold different. GeXIVA[1,3] and GeXIVA[1,4] showed equal potency for $\alpha 6/\alpha 3\beta 2\beta 3$, with IC_{50} s of 149 nM and 199 nM, respectively. GeXIVA[1,2] was 2.6-fold less potent than GeXIVA[1,4], with an IC_{50} of 508 nM. On the other hand, GeXIVA[1,2] was 25-fold more potent at the $\alpha 9\alpha 10$ nAChR than at $\alpha 7$, $\alpha 1\beta 1\delta\epsilon$ and $\alpha 6/\alpha 3\beta 2\beta 3$. GeXIVA[1,3] showed similar activities on $\alpha 9\alpha 10$ and $\alpha 6/\alpha 3\beta 2\beta 3$, with close IC_{50} s; however, it was only weakly potent on $\alpha 7$ – 31-fold less potent than $\alpha 9\alpha 10$. GeXIVA[1,4] was around 4–19-fold more potent at the $\alpha 9\alpha 10$ nAChR than $\alpha 7$, $\alpha 1\beta 1\delta\epsilon$ and $\alpha 6/\alpha 3\beta 2\beta 3$. All three isomers of GeXIVA retained selectivity for $\alpha 9\alpha 10$ but not the other nAChR subtypes.

3.4. Effect of the isomers of GeXIVA on $\alpha 9\alpha 10$ nAChR mutants

Mutations within the N-terminal ligand-binding domain of the $\alpha 9$ and $\alpha 10$ subunits of the rat nAChR were used to probe the binding site of GeXIVA. The activity of 10 nAChR $\alpha 9$ and $\alpha 10$ mutants ($\alpha 9T56I\alpha 10$, $\alpha 9S6N\alpha 10$, $\alpha 9\alpha 10E61S$, $\alpha 9\alpha 10D121L$, $\alpha 9S117A\alpha 10$, $\alpha 9S14N\alpha 10$, $\alpha 9S136N\alpha 10$, $\alpha 9A24K\alpha 10$, $\alpha 9R71G\alpha 10$ and $\alpha 9E192Q\alpha 10$) were measured for various concentrations of the three isomers (Table 3, Fig. 4). All three isomers potently blocked the 10 $\alpha 9\alpha 10$ nAChR mutants, and only small differences in affinity were observed; IC_{50} s were similar to those seen for $\alpha 9\alpha 10$ and although the isomers were less potent than at wild-type $\alpha 9\alpha 10$ nAChRs, they were still within a 10-fold difference. The most potent isomer, GeXIVA[1,2], blocked the 10 $\alpha 9\alpha 10$ mutants with IC_{50} values ranging from 19.5 to 59.3 nM. IC_{50} s for GeXIVA[1,4] ranged from 24 to 96 nM, and from 68–232 nM for GeXIVA[1,3]. The order of potency of the isomers, in terms of the $\alpha 9\alpha 10$ mutants was [1,2] > [1,4] > [1,3], identical to the trend for inhibition of the rat and human wild-type receptors.

Most of the mutations involved residues of the rat receptor being substituted by those of the human receptor, and the absence of measurable effects of these mutations is consistent with the very similar activity of the peptides whatever the origin of the receptor. Fig. 5 shows the location of the mutated positions on the ligand-binding domain of $\alpha 9\alpha 10$ nAChR, highlighting that the selected positions cover a large area of the receptor. Because these positions are widespread and because the mutations caused similar changes of activity, the impacts of the mutations on activity are not highly definitive. The positions that were mutated might therefore either not be part of the interface with the peptide ligands, or might be at the peptide binding interface but with the particular amino acids chosen for mutation not measurably impacting the binding affinity.

Fig. 5 also displays the location of the two potential binding modes that were proposed previously for GeXIVA[1,4] (Luo et al., 2015). Only the two mutations $\alpha 9S6N$ and $\alpha 10D121L$ interrogate the proposed models, but mutations of these residues in the models lead to no change of hydrogen bonding and to a smaller than 4 Å² difference in contact surface area. The buried surface areas were 2720 Å² and 1700 Å² for the binding mode in the agonist binding site and the site at the outskirts of the pore, respectively.

The mutations therefore cannot help prove or disprove the previously proposed models. Mutated positions $\alpha 9T56$ and $\alpha 9S117$ are located in the orthosteric binding site $\alpha 10(+)\alpha 9(-)$, which is distinct from the previously proposed binding mode of GeXIVA[1,4] in the $\alpha 9(+)\alpha 10(-)$ orthosteric site or in the pore. It was previously suggested that GeXIVA [1,2] did not compete for binding with conotoxin RgIA (Luo et al., 2015), which binds to the $\alpha 10(+)\alpha 9(-)$ binding site (Azam et al., 2015). The mutation $\alpha 9T56I$ was shown to impact the activity of RgIA (Azam et al., 2015), but the same mutation here had no measurable effect, further supporting the idea that the GeXIVA isomers do not bind to the $\alpha 10(+)\alpha 9(-)$ binding site.

4. Discussion

Both the bead and ribbon isomers of α O-CTx GeXIVA have previously been shown to significantly reduce mechanical hyperalgesia in the rat chronic constriction injury model of neuropathic pain without affecting motor performance (Li et al., 2016; Luo et al., 2015). In those studies, the analgesic effects of GeXIVA[1,2] and GeXIVA[1,4] occurred at low doses (2 nmol) without tolerance issues. Although, in both cases, a 2 nmol intramuscular dose produced effects greater than or equal to that observed with 500 nmol of the opioid analgesic morphine (Li et al., 2016; Luo et al., 2015), Li and colleagues observed the analgesic effects of GeXIVA[1,2] to be slightly better than GeXIVA[1,4] (Li et al., 2016). Furthermore, they reported that the pain relieving effect of GeXIVA[1,2] remained for up to 2 weeks after cessation of drug administration. Like GeXIVA, the α -CTxs RgIA and Vc1.1 are known to be potent antagonists of α 9 α 10 nAChRs, and their potential as human therapeutics is being investigated (Lewis et al., 2012; Livett et al., 2006; McIntosh et al., 2009; Vincler et al., 2006). When tested in rat models of neuropathic pain, both Vc1.1 and RgIA induced analgesia (Azam and McIntosh, 2012). However, when human and rat nAChRs were compared, RgIA was found to be over 300-fold less potent at the h α 9 α 10 nAChR (Table 4) (Azam and McIntosh, 2012) whereas Vc1.1 was found to be around 400-fold less potent at the h α 9 α 10 nAChR compared to the rat receptor (Table 4) (Luo et al., 2015). A molecular basis for the differential sensitivity of rat and human α 9 α 10 nAChRs to RgIA was investigated (Azam and McIntosh, 2012), and it was found that the primary determinant of this disparity is a single amino acid – a Thr56 (rat) to Ile56 (human). The current study aimed to further explore differences between rat and human receptors.

Vc1.1 reached phase 2A human clinical trials but trials were halted with the discovery that Vc1.1 had lower affinity for the h α 9 α 10 nAChR compared to the r α 9 α 10 nAChR (<http://www.evaluategroup.com/Universal/View.aspx?type=Story&id=135083>, Lewis et al., 2012) and in the absence of a clear efficacy signal. Subsequent reports indicated that Vc1.1 has potent GABA_B agonist activity (Berecki et al., 2014; Callaghan and Adams, 2010; Callaghan et al., 2008; Castro et al., 2016; Cuny et al., 2012; Huynh et al., 2015; Klimis et al., 2011; Nevin et al., 2007; van Lierop et al., 2013). These findings have prompted an ongoing debate as to whether the analgesic mechanism of action of the α -conotoxins is via α 9 α 10 nAChRs and/ or GABA_B receptors (Adams and Berecki, 2013; Mohammadi and Christie, 2014; Mohammadi and Christie, 2015; Wright et al., 2015). The pharmacological profile of GeXIVA coupled with its demonstrated analgesia in rat models of neuropathic pain has prompted further interest in α 9 α 10 nAChRs and GeXIVA as a potential therapeutic agent (Luo et al., 2015; Li et al., 2016).

The present study supports a potential application of GeXIVA isomers in the treatment of neuropathic pain. All three isomers of GeXIVA have similar activities at the human and rat α 9 α 10 nAChRs, with smaller than 5-fold differences (Table 4). They have mid- to low-nanomolar IC₅₀ values (19.6–80.6 nM) for the rat α 9 α 10 mutant r α 9T56I α 10, which are similar to the values for the wild-type rat and human α 9 α 10 receptors. In contrast, the IC₅₀ of α -RgIA for r α 9T56I α 10 is 2560 nM – 1700-fold less than for r α 9 α 10. The potency of α D-CTx GeXXA (Xu et al., 2015) is 23.3-fold less for the human α 9 α 10 receptor compared to the rat α 9 α 10 receptor (Table 4). Thus, among all of the aforementioned peptides,

GeXIVA[1,2] has the greatest affinity for $\alpha 9\alpha 10$ nAChRs (IC_{50} : 20 nM). This finding is consistent with the binding site of GeXIVA on rat and human $\alpha 9\alpha 10$ receptors being novel and distinct from that of α -RgIA, α -Vc1.1 and α D-GeXXA.

α -Conotoxins are competitive blockers of nAChRs, with the blocking site of $\alpha 9\alpha 10$ nAChRs located at the $\alpha 10/\alpha 9$ subunit interface. A species difference renders these conotoxins orders of magnitude less potent on the human vs. rat nAChR (Azam and McIntosh, 2012; Azam et al., 2015; Yu et al., 2013). In the present study, rat $\alpha 9\alpha 10$ nAChR mutants were used to compare the binding properties of the three isomers of GeXIVA (Fig. 4, Table 3) to those established for other conotoxins. All of the mutated rat $\alpha 9\alpha 10$ receptors were found to have a similar affinity for the GeXIVA isomers as the wild-type rat and human $\alpha 9\alpha 10$ nAChRs, with a difference of < 14-fold for rat and < 3-fold for human. This finding confirms that GeXIVA binds different regions of $\alpha 9\alpha 10$ than those identified in α -CTx RgIA and Vc1.1 and α D-CTx ligand-binding studies. Consistent with this, a previous study indicated that block by GeXIVA[1,2] was strongly voltage-dependent, suggesting that the toxin binding site might be allosterically coupled to a voltage-sensitive domain of the nAChR (Luo et al., 2015).

α -Conotoxins with a CC-C-C cysteine framework typically comprise 12–20-amino acids and display a globular disulfide connectivity linking Cys 1 to 3, and 2 to 4. The alternative connectivities – ribbon (Cys1–4, 2–3) and bead (Cys1–2, 3–4) – are generally associated with lower potency. As an example, the native globular isomer of α -conotoxin GI is active at mammalian muscle nAChRs with full biological activity; however, the bead isomer is 10-fold less potent and the ribbon isomer has little or no biological activity (Gehrmann et al., 1998). The ribbon α -GI isomer more readily rearranges to the native globular isomer than the bead isomer, according to stability studies in various assay buffers (Armishaw et al., 2010). In one case, conotoxin α -AuIB, an alternative isomer was shown to be more active at the $\alpha 3\beta 4$ receptor if the receptor contains three $\alpha 3$ and two $\beta 4$ subunits, but not if the pentamer is formed by assembly of two $\alpha 3$ and three $\beta 4$ subunits (Grishin et al., 2010). The ribbon disulfide isomer α -AuIB has a less well-defined structure than the globular form (Dutton et al., 2002), and the unexpected activity of the ribbon isomer α -AuIB may be ascribed to the structural flexibility of the molecule, which may allow for a more complementary fit in the binding site.

A disulfide bond between adjacent cysteines is energetically unfavorable, as is the case for the α B-CTx VxXXIVA which has a C-CC-C framework. In the case of GeXIVA, the bead isomer with the 1–2, 3–4 connectivity (GeXIVA[1,2]) blocks rat $\alpha 9\alpha 10$ nAChRs, and is 5~6 fold more active than the 1–3, 2–4 (GeXIVA[1,3]) form. VxXXIVA is atypical among disulfide-bridged conotoxins because it has disordered structures in aqueous solution over a range of temperatures and pHs (Luo et al., 2013). In the current study, all three isomers of the GeXIVA (C-C-C-C framework), blocked rat and human $\alpha 9\alpha 10$ nAChRs, with the order of potency being bead (Cys1–2, 3–4, GeXIVA [1,2]) > ribbon (Cys1–4, 2–3, GeXIVA [1,4]) > globular (Cys1–3, 2–4, GeXIVA [1,3]) (Table 5). In addition, the three isomers of GeXIVA exhibited differential sensitivity to various nAChRs (Table 1–4). The order of the isomers remained the same for rat and human $\alpha 9\alpha 10$ and $\alpha 7$ subtypes as GeXIVA, which was also similar ([1,2] $\approx$ [1,3] $\approx$ [1,4]) for the $\alpha 1\beta 1\delta\epsilon$, mouse $\alpha 1\beta 1\delta\epsilon$, $\alpha 6/\alpha 3\beta 2\beta 3$ and

ra.3β2 subtypes. For the ha.6/α.3β2β3, ra.2β4, ra.4β4 and ra.6/α.3β4 subtypes, the order of potency of the isomers differed, with [1,3] ≈ [1,4] >> [1,2]; that is to say, the bead isomer GeXIVA[1,2] was the least potent, and the globular and ribbon isomers, GeXIVA[1,3] and [1,4], respectively, had similar potency. The different sensitivities to different nAChRs subtypes may be attributed to the three-dimensional structures of the isomers and corresponding receptors and their interactions. Although both the αO-CTx GeXIVA and α-conotoxins more generally contain four Cys residues, their frameworks are very different. αO-CTx GeXIVA has a 'four separate' Cys pattern (C-C-C-C), whereas α-conotoxins and αB-VxXXIVA have a 'two adjacent and two separate' Cys pattern (CC-C-C: α-CTx; C-CC-C: αB-CTx) (Table 5). The difference in the functions of the disulfide bond isomers is reflected in the differences between their sequences, Cys arrangements, and three-dimensional structures.

The α9α10 nAChR is an important target for the development of analgesics and cancer chemotherapeutics (Chen et al., 2011; Chikova et al., 2012; Klimis et al., 2011; Satkunathan et al., 2005; Wu et al., 2011). The design and development of α*-conotoxin analogs with improved potency at human α9α10 nAChRs and that maintain selectivity over other nAChR subtypes, may especially assist in the treatment of neuropathic pain (Halai et al., 2009). The three isomers of GeXIVA have a unique selectivity among conotoxins as they potently inhibit both rat and human α9α10 nAChRs, potentially allowing a better translation of effects from animal models to the clinic.

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Abbreviations

CTx	conotoxin
nAChRs	nicotinic acetylcholine receptors
ha.9α10	human α9α10
ra.9α10	rat α9α10

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Highlights

- α O-conotoxin GeXIVA is a potent antagonist of rat $\alpha 9\alpha 10$ nAChRs.
- Three disulfide bond isomers of GeXIVA have differential sensitivity for various nAChRs.
- The effects of the three isomers on 10 rat $\alpha 9\alpha 10$ nAChR mutants are similar.
- The isomers retain potency and selectivity for the human $\alpha 9\alpha 10$ subtype.
- The 'bead' isomer has the greatest affinity for h $\alpha 9\alpha 10$ nAChRs.

Fig. 1.

Percentage inhibition of the mouse muscle type $\alpha 1\beta 1\delta\epsilon$ nAChR and rat $\alpha 7$, $\alpha 2\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 6/\alpha 3\beta 2\beta 3$ and $\alpha 6/\alpha 3\beta 4$ nAChR subtypes by various concentrations of the three α O-GeXIVA isomers. Values are mean $\pm$ SEM from 3–6 separate oocytes. IC_{50} values corresponding to each curve are provided in Table 1.

Fig. 2.

The three α O-GeXIVA isomers block human $\alpha 9\alpha 10$ nAChR differently at 100 nM concentration. (A) Bead α O-GeXIVA[1,2], (B) globular α O-GeXIVA[1,3], (C) ribbon α O-GeXIVA[1,4]. Oocytes expressing human $\alpha 9\alpha 10$ nAChR were voltage clamped at -70 mV and subjected to a 1 second pulse of $10 \mu\text{M}$ ACh every minute as described in the Materials and Methods. In panel A, B and C, the initial peaks are control responses, following which the oocyte was exposed to the peptide for 5 min. ND96 perfusion and ACh pulses were then resumed to monitor recovery from toxin block. The arrow denotes the current response trace on $\alpha 9\alpha 10$ nAChRs in the presence of 100 nM peptide. The bead isomer α O-GeXIVA[1,2] was most potent as it nearly completely blocked human $\alpha 9\alpha 10$ nAChR at 100 nM, whereas the globular α O-GeXIVA[1,3] was the least potent with only 43% block; the ribbon α O-GeXIVA[1,4] had an intermediate activity with 67% block at 100 nM and its recovery was slower than the other two isomers.

Fig. 3.

Concentration-response of the three α O-GeXIVA isomers on human α 9 α 10, α 7, α 1 β 1 $\delta\epsilon$ and α 6/ α 3 β 2 β 3 nAChR subtypes. Values are mean $\pm$ SEM from 5–9 separate oocytes. IC₅₀ values corresponding to each curve are provided in Table 2.

Fig. 4.

Concentration-response curves of the three α O-GeXIVA isomers for inhibition of rat α 9 α 10 nAChR mutants. Values are mean $\pm$ SEM from 3–5 separate oocytes. IC₅₀ values corresponding to each curve are provided in Table 3.

Fig. 5. Spatial location of the positions that have been mutated in the $\alpha 9$ and $\alpha 10$ subunits relative to the two proposed binding modes of GeXIVA on the $\alpha 9\alpha 10$ nAChR. The ligand binding domain of the $\alpha 9$ and $\alpha 10$ subunits chosen for illustration are represented using a cartoon representation in green and grey, respectively. The positions that have been mutated in these subunits are represented using spheres. The positions the substitution of which led to ~10-fold or ~5-fold decrease binding of GeXIVA bead isomer are in red and blue, respectively. The transmembrane domain is illustrated as rectangles. All $\alpha 9$ subunits are in green and $\alpha 10$ subunits in grey. The two binding modes of GeXIVA that have been proposed previously are located in the pore and in the orthosteric binding $\alpha 9(+)\alpha 10(-)$. Ribbon GeXIVA is colored in orange.

Table 1
IC₅₀ and Hill slope values for block of rat nAChR subtypes by αO-CTx GeXIVA[1,2], [1,3] & [1,4].

Subtypes	GeXIVA[1,2] ^b			GeXIVA[1,3]			GeXIVA[1,4]		
	IC ₅₀ (nM) ^a	Hill slope ^a		IC ₅₀ (nM) ^a	Hill slope ^a		IC ₅₀ (nM) ^a	Hill slope ^a	
ra9α10	4.61 (3.18–6.65)	0.56 (0.44–0.69)		22.7(11.8–43.5) ^b	0.78(0.29–1.26) ^b		7.0(3.6–13.4) ^b	0.79(0.23–1.36) ^b	
ra7	432(290–644)	1.10(0.72–1.48)		4990(4264–5840)	1.72(1.25–2.18)		1768(1327–2354)	1.00(0.70–1.29)	
Mouse α1β1δε	394(311–498)	1.71(0.98–2.43)		683(543–858)	1.47(0.91–2.02)		484(399–587)	1.64(1.15–2.14)	
ra6/α3β2β3	449(355–569)	0.69(0.59–0.79)		369(191–710)	0.69(0.41–0.98)		555(246–1252)	0.64(0.34–0.94)	
ra3β2	480(362–638)	0.91(0.67–1.14)		613(432–869)	1.48(0.73–2.23)		489(308–777)	1.06(0.63–1.49)	
ra2β2	499(379–656)	1.23(0.91–1.55)		348(237–511)	1.19(0.80–1.57)		122(89.7–166)	0.95(0.70–1.20)	
ra4β2	1350(960–1897)	0.99(0.70–1.23)		1261(532–2986)	0.63(0.27–0.99)		215(132–352)	0.82(0.53–1.11)	
ra2β4	5523(4600–6632)	1.03(0.80–1.26)		1283(1079–1524)	1.16(0.92–1.39)		1082(911–1285)	1.33(1.01–1.65)	
ra4β4	2512(1630–3870)	0.79(0.51–1.08)		1100(795–1521)	1.33(0.72–1.94)		869(675–1118)	1.18(0.81–1.55)	
ra6/α3β4	832(718–965)	1.34(0.95–1.74)		497(400–616)	1.75(1.29–2.21)		634(474–821)	1.39(0.84–1.94)	
ra3β4	5409(3536–8274)	1.13 (0.53–1.74)		2003(1042–3850)	0.88(0.31–1.45)		3396(2394–4817)	1.06(0.62–1.49)	

^aNumbers in parentheses are 95% confidence intervals;

^bData from Luo et al., 2015(Luo et al., 2015).

IC₅₀ and Hill slope values for block of human nAChR subtypes by αO-CTx GeXIVA[1,2], [1,3] & [1,4].

Table 2

Subtypes	αO-GeXIVA[1,2]			αO-GeXIVA[1,3]			αO-GeXIVA[1,4]		
	IC ₅₀ (nM) ^a	Hill slope ^a	IC ₅₀ (nM) ^a	Hill slope ^a	IC ₅₀ (nM) ^a	Hill slope ^a	IC ₅₀ (nM) ^a	Hill slope ^a	Hill slope ^a
ha9α10	20.3(12.4–33.2) ^a	0.91(0.49–1.32) ^a	116(65–204)	0.73(0.45–1.01)	47.3(29.7–75.3)	0.67(0.46–0.88)			
ha7	559(429–728)	1.17(0.85–1.49)	3584(2476–5186)	0.96(0.60–1.32)	889(739–1068)	1.96(1.05–2.87)			
ha1β18e	497(391–633)	1.65(1.02–2.27)	485(398–592)	1.62(1.11–2.13)	365(279–477)	1.80(0.88–2.72)			
ha6/α3β2β3	508(345–747)	0.95(0.65–1.24)	149(112–200)	1.00(0.69–1.32)	199(134–295)	1.11(0.68–1.54)			

^aNumbers in parentheses are 95% confidence intervals

Table 3

IC₅₀ (nM)^a values for block of the rat $\alpha 9\alpha 10$ nAChR mutants by each isomer of α O-CTx GeXIVA.

Subtype/mutant	GeXIVA[1,2]	GeXIVA[1,3]	GeXIVA[1,4]
ra9a10	4.61 (3.18–6.65)^a	22.7 (11.8–43.5)^a	7.0 (3.6–13.4)^a
ha9a10	20.3(12.4–33.2)^a	116(65.4–204)	47.3(29.7–75.3)
ra9S6Na10	19.8 (13.7–28.6)	119 (79.8–178)	56.8 (32.4–99.6)
ra9S14Na10	52.5 (45.3–60.8)	123 (65.2–231)	59.7 (51.0–69.9)
ra9A24Ka10	51.6 (42.0–63.5)	170 (82.5–350)	96.0 (66.1–139)
ra9T56la10	19.6(14.2–26.9)	80.6 (64.4–101)	29.5 (20.1–43.2)
ra9R71Ga10	59.3 (44.2–79.6)	232 (160–337)	61.6 (47.4–79.9)
ra9S117Aa10	32.1 (21.6–47.8)	182 (87.5–379)	46.7 (7.63–286)
ra9S136Na10	45.7 (41.3–50.6)	106 (75.5–149)	33.7 (28.4–40.0)
ra9E192Qa10	49.6 (35.1–70.3)	143 (91.7–224)	63.2 (43.7–91.5)
ra9a10E61S	25.1 (17.7–35.4)	67.9 (52.2–88.2)	23.9 (16.7–34.1)
ra9a10D121L	27.6 (18.3–41.7)	122 (67.4–220)	45.1 (22.8–89.4)

^aIC₅₀ (nM), Numbers in parentheses are 95% confidence intervals.

Comparison of the IC₅₀ values of αO- GeXIVA isomers, α- RgIA, α- Vc1.1, and αD-GeXXA at rat and human α9α10 nAChRs and at the ra9T56fa.10 mutant.

Peptide	ra9α10 [IC ₅₀ (nM)]	ha9α10 [IC ₅₀ (nM)]	Ratio ^a (human /rat)	ra9T56fa.10 [IC ₅₀ (nM)]	Ratio ^b (ra9T56fa.10/ ra9α10)
αO-CTx GeXIVA[1,2]	4.61	20	4.3	19.5	4.2
αO-CTx GeXIVA[1,3]	22.7	116	5.1	80.6	3.6
αO-CTx GeXIVA[1,4]	7	47	6.7	29.5	4.2
α-CTx RgIA[10]	1.49	494	332	2560	1718
α-CTx Vc1.1	19 ^c	7500	395	NA	NA
αD-CTx GeXXA	1.2	28	23.3	NA	NA

^ahuman α9α10 nAChR subtype IC₅₀/ rat α9α10 IC₅₀.

^brat α9T56fa.10 nAChR mutant IC₅₀/rat α9α10 IC₅₀.

^cData from Vincler et al., 2006(Vincler et al., 2006).
NA: not available.

Table 5

Comparison of the activity of the disulfide isomers of a selection of conotoxins with four cysteines and belonging to different gene superfamilies.

Name	Gene super family	Sequence	Length in amino acids	nAChR subtype	Isomer IC ₅₀ nM (activity)			Potency ranking of isomers	Reference
					B[1,2] ^a	G[1,3] ^b	R[1,4] ^c		
αO-GeXIVA	O	T R S S G R Y R S P Y D R R R R Y R R R I T D A C V	28	ra9α10 ^d ha9α10	4.6 20	23 116	7 47	[1,2]>[1,4]>[1,3]	This work
αB-VαXXIVA	B	V R C L E K S G A Q P N K L F R P P Q K G P S F A R H S R C V Y Y T Q S R E	40	ra9α10	1200	3900	>30000	[1,2]>[1,3]>[1,4]	(Luo et al., 2013)
α-AuIB	A	G S Y P P C F A T N P D C #	15	ra3β4 (1:10) ^e ra3β4 (10:1)	n.d. n.d.	3000 1100	NA 860	[1,3]>[1,4] [1,4]>[1,3]	(Grishin et al., 2010) (Grishin et al., 2010)

= C-terminus amidation,

^aB[1,2] or [1,2] is the “bead” isomer with a disulfide connectivity Cys1–Cys2, Cys3–Cys4 (C1–2, C3–4);

^bG[1,3] or [1,3] is the “globular” isomer with a disulfide connectivity Cys1–Cys3, Cys2–Cys4 (C1–3, C2–4);

^cR[1,4] or [1,4] is the “ribbon” isomer with a disulfide connectivity Cys1–Cys4, Cys2–Cys3 (C1–4, C2–3).

^dData from Luo et al., 2015(Luo et al. 2015). E ratio of quantity of α3 and β4 mRNA injected in oocytes.

^eratio of quantity of α3 and β4 mRNA injected in oocytes.

Abbreviations: ra9α10 and ra3β4 means rat α9α10 and α3β4; ha9α10 means human α9α10; n.d. is “not determined”; and NA is “not active”.