μ-Conotoxin KIIIA peptidomimetics that block human voltage-gated sodium channels

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Abstract
Peptidomimetics designed to target voltage-gated sodium channels have attracted significant attention as potential analgesics. However, voltage-gated sodium channel (VGSC)-blocking activity of these compounds has mainly been assessed using rat and/or mice homologs. In this study, we developed a novel series of conformationally constrained peptidomimetic analogues of the μ-conotoxin KIIIA and assessed their activity against human VGSCs. Two of the mimetics block the currents of hNa\textsubscript{v}1.4 and hNa\textsubscript{v}1.6 channels. NMR derived structures of the mimetics provided excellent insight into the structural requirements for bioactivity. A lactam-constrained analogue, previously reported to be active in mice, did not block the corresponding human VGSC. This work highlights important differences in VGSCs between species and validates the potential of peptidomimetics as human analgesics.

1 | INTRODUCTION

Conotoxin peptides are isolated from the venom of fish hunting marine snails, which use them as an efficient means of prey incapacitation and as an effective defense mechanism. Conotoxin venom has been reported to have caused several human fatalities\cite{1-3} and can pose a risk to human health.\cite{4} However, conotoxins and their synthetic mimetics have attracted a substantial amount of interest due to their properties as selective chemical probes that target ion channels and receptors.\cite{5-8} They are potential therapeutic leads for several clinical applications including the treatment of neuropathic pain, hypertension and type 2 diabetes.\cite{9} Ziconotide, a synthetic analogue of the conotoxin peptide ω-MVIIA is licensed for clinical use in the treatment of severe and chronic pain.\cite{10} Due to unfavorable physicochemical properties of the native peptides the development of conotoxin peptidomimetics has been an active field of study by academics and the pharmaceutical industry.\cite{11} Development of new peptidomimetic chemistries that can be used to overcome the physicochemical limitations of native peptides and provide peptide leads for drug discovery are a high priority. This is generally achieved by chemically modifying the peptide structure, removing the vulnerable functionalities and introducing mimetic features to retain the overall three-dimensional bioactive conformation of the peptide.

μ-Conotoxin KIIIA (μ-KIIIA) is isolated from the venom of the marine cone snail Conus kinoshitai.\cite{12} The peptide primary sequence contains 16 amino acid residues that are constrained by three disulfide bonds to adopt an α-helical secondary structure. As with other...
μ-conotoxins, μ-KIIIA modulates the activity of voltage-gated sodium channels (VGSCs) by occluding the pore in the middle of the channel. VGSCs are heterotrimetric protein complexes composed of one α-subunit and two β-subunits that form a single pore. The ion-conducting core subunits (α-subunits) are solely required for voltage-dependent ion permeation, and the β-subunits are responsible for regulating membrane trafficking and channel properties.[13] Nine different subtypes of the α-subunit of VGSCs have been identified in humans: Na\textsubscript{v}1.1-Na\textsubscript{v}1.9. Subtypes Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, and Na\textsubscript{v}1.6 are primarily located in the central nervous system (CNS). Na\textsubscript{v}1.4 functions in skeletal muscle and Na\textsubscript{v}1.5 in the heart. Na\textsubscript{v}1.7, Na\textsubscript{v}1.8, and Na\textsubscript{v}1.9 are mainly found in the peripheral nervous system.

The first structure of a human VGSC (Na\textsubscript{v}1.4-β1 complex) was solved to 3.2 Å resolution using cryo-electron microscopy in 2018.[14] This was followed in 2019 by the cryo-EM structure of μ-KIIIA bound to human VGSC Na\textsubscript{v}1.2.[15] The cryo-EM structure of human Na\textsubscript{v}1.7 in complex with auxiliary subunits and animal toxins has also recently been reported.[16] These structures provide detailed information on the molecular mechanisms of VGSC blocking by peptide ligands and may potentially facilitate VGSC structure-based drug discovery.

Subtype Na\textsubscript{v}1.7 has been identified as a therapeutic target for the treatment of pain through genetic association with gain-of-function mutations in the SCN9A gene as the basis of inherited pain syndromes, including erythromelalgia and paroxysmal extreme pain disorder.[17] μ-KIIIA has rank order selectivity of Na\textsubscript{v}1.4 (48 ± 6 nM) ≥ Na\textsubscript{v}1.2 (61 ± 5 nM) > Na\textsubscript{v}1.6 (183 ± 31 nM) > Na\textsubscript{v}1.3 (3.6 ± 0.3 μM), and has no effect on Na\textsubscript{v}1.5 and Na\textsubscript{v}1.8 (at 10 μM).[18]

Numerous peptidomimetic strategies have been applied to μ-conotoxins including peptide sequence miniaturization,[19] N- and C-terminal extension,[20] chimeras,[21] and small molecule mimetics.[22] Previous research by Norton and co-workers examined the effect of μ-KIIIA peptidomimetics, incorporating a lactam conformational constraint, on the VGSC subtypes Na\textsubscript{v}1.1-1.9.[23] A [D9K13]-lactam analogue was identified with an IC\textsubscript{50} of 54 μM against rat Na\textsubscript{v}1.4 and 40 μM against mouse Na\textsubscript{v}1.6.[23]

This synthetic peptidomimetic strategy provides ready access to analogues to determine structure-activity-relationships and information on key functionalities required for the selective interaction with this important class of therapeutic target.[24] However, one limitation of previous studies is the use of rat and/or mouse sodium channels. Although rodent and human voltage-gated sodium channels have a high degree of amino acid sequence homology (ex. 92% sequence homology between human and rat Na\textsubscript{v}1.4), their sensitivity to ligands often differs between species.[24] The origin of these differences in many cases has yet to be elucidated, yet is likely to be revealed as more ion-channel structures are solved. The use of human VGSCs, expressed in human cells would give the most relevant data to underpin and expedite a medicinal chemistry programme.

The aim of the present work was to investigate whether synthetic conformational constraints could replace the complex disulfide bond bridging network in the μ-KIIIA conotoxin peptide, and produce more stable analogues that would retain bioactivity against human VGSCs.

2 | MATERIALS AND METHODS

2.1 | General information

Standard Fmoc-protected amino acids were purchased from CEM Corporation or Pepceuticals and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), peptide grade dimethyl formaldehyde (DMF) and ethyl (hydroxyimino) cyanoacetate (Oxyma Pure) were purchased from Pepceuticals. (R)-N-Fmoc-α-(7-octenyl)alanine was purchased from Nagase Europe (GmbH) and (S)-N-Fmoc-α-(4-pentenyl)alanine was purchased from Sigma Aldrich. Fmoc-Asp(O-2-PhPr)-OH and Fmoc-Lys(Mmt)-OH were purchased from Merck. 2,4,6-Trisopropylbenzenesulfonyl hydrazide (TPSH) was purchased from TCI Chemicals. All other reagents were purchased from Sigma Aldrich.

Flash column chromatography was performed on a Biotage Isolera using prepacked KP-SIL silica gel SNAP cartridges (50 μm). Thin layer chromatography was run on aluminium-backed plates precoated with silica gel 60F\textsubscript{254} (Merck) and spots were visualized with a UV lamp or stained with ninhydrin. Spectra were recorded on a 400, 500, or 600 MHz Bruker NMR spectrometer, and chemical shifts are reported in parts per million and corrected relative to the solvent as internal standard (CDCl\textsubscript{3} 6.26). Multiplicity is reported as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad singlet. IR was run on a Nicolet i55 from Thermo Fisher mounted with an iD5 ATR. Optical rotation was measured on an Autopol V automatic polarimeter (Rudolph Research Analytical) using the sodium D line at 589 nm.

Peptides were synthesized on a Biotage Initiator + Altra microwave assisted peptide synthesizer. Reactions requiring microwave heating were performed using a CEM Discover SP microwave. Peptides were purified on a reverse-phase Dionex high performance liquid chromatography (HPLC) system equipped with Dionex P680 pumps and a Dionex UVD170U UV-vis detector (monitoring at 214 nm and 280 nm), using a Phenomenex Gemini, C18, 5 μm, 250 x 21.2 mm column. Gradients were run using a solvent system consisting of A (H\textsubscript{2}O+0.1% TFA) and B (H\textsubscript{2}O + 0.1% TFA), and collected fractions were lyophilised on a Christ Alpha 2.4 LO plus freeze dryer.

Pure peptides were analyzed on a Shimadzu reverse-phase HPLC (RP-HPLC) system equipped with Shimadzu LC-20AT pumps, a Shimadzu SIL-20A autosampler and a Shimadzu SPD-20A UV-vis detector (monitoring at 200 nm and 280 nm) using a Phenomenex, Aeris, 5 μm, peptide XB-C18, 150 x 4.6 mm column at a flow rate of 1 mL/min. RP-HPLC gradients were run using a solvent system consisting of solution A (5% acetonitrile [MeCN] in H\textsubscript{2}O + 0.1% TFA) and B (5% H\textsubscript{2}O in MeCN +0.1% TFA). Two gradients were used to characterize each peptide; a gradient from 0% to 100% solution B over 20 minutes and either a 50 minutes gradient from 0%-100% solution B or a 25 minutes gradient from 0-50% solution B, respectively. Analytical RP-HPLC data is reported as column retention time (t\textsubscript{R}) in minutes (min). Low resolution mass spectrometry (LRMS) was performed on a Thermo Scientific LCQ Fleet quadrupole mass spectrometer using positive mode electrospray ionization (ESI\textsuperscript{+}). High resolution mass spectrometry (HRMS) was performed on a Bruker
microTOF-Q II (ESI+). HRMS data are reported as mass to charge ratio 
(m/z) = observed / MW.

Peptide content was analyzed on a Nanodrop 2000c using UV absorption of peptides at 280 nm.

2.2 | Peptide synthesis

Peptides were synthesized on 0.1 mmol scale using ChemMatrix Rink Amide Resin (0.47 mmol/g) (Biotage). Couplings were performed using 4 equivalents Fmoc-protected amino acid, 4 equivalents HCTU and 4 equivalents diisopropylethylamine (DiPEA) in DMF (3 mL). Coupling of standard Fmoc-protected amino acids was carried out for 10 minutes at 75 °C followed by 4 × 45 seconds washes. Arginine was double coupled: 60 minutes at room temperature followed by 5 minutes at 75 °C and repeated with fresh reagents followed by washing. Histidine and cysteine were coupled at room temperature for 5 minutes followed by 50 °C for 5 minutes and washes. For coupling of unnatural amino acids only 2 equivalents of reagents were used.

Deprotection was carried out in 20% piperidine in DMF + 0.1 M Oxyma Pure (4.5 mL) for 30s and then 3 minutes at 75 °C followed by washing. Following coupling of Fmoc-Asp(tBu)-OH all Fmoc-deprotections were carried out at room temperature for 3 minutes and then 10 minutes followed by washing. N-terminal acetylation was performed using 1 mL 5 M acetic anhydride in DMF and 2.5 mL 2 M DiPEA in NMP for 10 minutes at room temperature.

Test cleavages were performed in a cleavage cocktail (1 mL) of 95% trifluoroacetic acid (TFA), 2.5% water (H₂O) and 2.5% trisopropylsilane (TIS) for 30 minutes at room temperature and the cleavage cocktail was evaporated using a stream of nitrogen. The peptide was precipitated from solution with ice-cold diethyl ether (Et₂O), centrifuged at 4500 rpm for 5 minutes and the precipitate was dissolved in 50/50 MeCN/H₂O, centrifuged at 4500 rpm for 5 minutes and run using LC-MS and/or analytical HPLC.

Peptides containing cysteines were cleaved in a cleavage cocktail (10 mL) of 94% TFA, 2.5% 1,2-ethanediethiol (EDT), 2.5% H₂O and 1% TIS. Peptides without cysteines were cleaved in a cleavage cocktail (10 mL) of 95% TFA, 2.5% H₂O and 2.5% TIS. The resin was stirred for 3 hours at room temperature and the cleavage cocktail was evaporated using a stream of nitrogen. The peptides were precipitated from solution with ice-cold Et₂O, centrifuged at 4500 rpm for 5 minutes and the precipitate washed with ice cold Et₂O. The peptide was dissolved in H₂O/MeCN + a few drops of glacial acetic acid and lyophilised overnight.

2.2.1 | Synthesis of the native μ-KIIIA peptides 1 and 2

The linear peptide was synthesized, cleaved from resin and purified as described above. Peptide content was determined and the peptide was oxidized at a concentration of 20 μM to avoid polymerization. The peptide was oxidized using air oxidation in a 0.1 M ammonium carbonate (NH₄HCO₃) buffer at pH 7.5, which was degassed using N₂ for 1 hour prior to use. The reaction was monitored using LC-MS, and typically showed full conversion after 2-3 hours. The solution was then freeze dried and the residual solid taken into MeOH, which was removed in vacuo at 40 °C to remove most of the NH₄HCO₃-salt. The remaining solid was then dissolved in a mixture of H₂O/MeCN and purified as specified above yielding both the major and the minor folded isomers.

2.2.2 | Synthesis of the hydrocarbon stapled peptides 3, 4, 5 and 6 using ring closing-metathesis

The linear peptide was synthesized using solid phase peptide synthesis (SPPS) as described above. The resin was suspended in dry dichloroethane (DCE) (0.025 M) and 20 mol% Grubbs first catalyst was added. The reaction was shaken for 2 hours at room temperature excluding light. Following 3 × 1 minute dichloromethane (DCM) washes the procedure was repeated. If full conversion had not been attained the procedure was repeated in the microwave at 40 °C. After the final RCM the resin was thoroughly washed (3 × 1 minute DMF, 3 × 1 minute isopropanol (PrOH, 3 × 1 minute DCM) before resin cleavage/global deprotection and purification as described above.

2.2.3 | Synthesis of the reduced hydrocarbon stapled peptide 5

Resin containing hydrocarbon stapled peptide was swollen in N-methyl-2-pyrrolidone (NMP) for 20 minutes, the solvent removed and a mixture of trisopropylbenzenesulfonyl hydrazide (TPSH) (270 mg, 0.9 mmol, 9 eq.) and piperidine (180 uL, 1.8 mmol, 18 eq.) in 1 mL NMP was added. The resin was kept at 48 °C for 2 hours. The procedure was repeated 4-5 times. The resin was then thoroughly washed (3 × 1 minute DMF, 3 × 1 minute iPrOH, 3 × 1 minute DCM) before resin cleavage/global deprotection and purification as described above.

2.2.4 | Synthesis of the 1,4-triazole constrained peptide 7

The linear peptide was synthesized using the azide- and alkyne α-methyl amino acids S8 and S9 (see synthesis below) and cleaved from resin as described above. The crude peptide was then dissolved in pure, degassed H₂O at 1 mg/mL concentration and 1 eq. sodium ascorbate (NaAsc), 1 eq. copper(Ⅱ) sulfate pentahydrate (CuSO₄·5H₂O) and 8 eq. DiPEA were added. The solution was stirred at room temperature overnight. The reaction was then lyophilised and purified as described above.

2.2.5 | Synthesis of the 1,5-triazole constrained peptide 8

The linear peptide was synthesized using the azide- and alkyne α-methyl amino acids S8 and S9 (see synthesis below). The resin was
dried for 24 hours in a desiccator before being taken into 2 mL dry DMF and degassed for 20 minutes using argon gas. A 20 mol% \([\text{Cp}^*\text{RuCl(COD)}]\) was added and the solution was degassed for a further 10 minutes. The resin was then reacted in a microwave at 60 °C for 6 hours. The resin was washed thoroughly (3 × 1 minute DMF, 3 × 1 minute iPrOH, 3 × 1 minute DCM) and cleaved/globally deprotected and purified as described above.

### 2.2.6 Synthesis of the lactam constrained peptide 9

The linear peptide was synthesized using SPPS as described above, except that for Asp3 the amino acid Fmoc-Asp(2-O-Ph)-OH was used and for Lys7 the amino acid Fmoc-Lys(Mmt)-OH was used. The resin was swollen in DCM and washed with a solution of 2% TFA in DCM until the wash solution did not show a strong yellow color. The resin was washed thoroughly (3 × 1 minute DMF, 3 × 1 minute iPrOH, 3 × 1 minute DCM). The lactam was coupled using 1 eq. HCTU and 3 eq. DIPEA for 2 hours at room temperature and the coupling procedure was repeated using fresh reagents. The resin was washed thoroughly (3 × 1 minute DMF, 3 × 1 minute iPrOH, 3 × 1 minute DCM) and cleaved/globally deprotected and purified as described above.

### 2.3 Circular dichroism

Circular dichroism was performed on a Jasco J-810 spectropolarimeter at 20 °C. Concentration was determined on a Nanodrop and samples of 50 μM and 100 μM were made in pure H₂O with a maximum of 1% MeCN (final concentration) added for solubility. Before running CD the concentrations were checked again. Each sample was run at two different concentrations (50 μM and 100 μM) with 0.2 nm increments, 10 nm/step and 2 seconds response time. Both concentrations were compared to ensure that no dimerization/oligomerisation had occurred. A control sample containing pure H₂O was subtracted and the graph smoothed.

The raw data was corrected to give mean residue ellipticity (MRE) according to the following equation:

\[
\text{MRE} = \left(\frac{E}{10^3}\right) / l \cdot c \cdot n
\]

Where E is ellipticity in mdeg; l is pathlength in mm; c is peptide concentration in μM; n is number of amide bonds in the peptide.

### 2.4 Patch-clamp assay

Human embryonic kidney (HEK-293) cells expressing human Naᵥ1.4 (SB Drug Discovery, Glasgow, UK) were cultured in minimum essential media (MEM) medium with heat-inactivated Foetal Bovine Serum (FBS) (10%) glutamine (2 mM) penicillin/streptomycin (50 units/mL), blasticidin (2 μg/mL) and genetin 418 (0.6 mg/mL). Cells were grown in a humidified 5% CO₂ incubator at 37 °C, grown to 70/80% confluence and passed every 3 to 4 days using TrypLE express. For electrophysiology experiments cells were dissociated with TrypLE then resuspended in Ex-Cell ACF CHO medium and allowed to recover with stirring for 45 minutes on the Q-patch.

Whole cell patch clamp experiments were performed on a QPatch-16 automated electrophysiology platform (Sophion, Ballerup, Denmark) using 16 channel QPlates. The extracellular solution (ECS) contained 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES and 10 mM glucose, pH 7.4. The intracellular solution (ICS) contained 140 mM CsF, 1 mM EGTA, 10 mM HEPES, 5 mM CsOH and 10 mM NaCl, pH 7.25. The holding potential was −90 mV and channels were activated with a depolarizing step to 0 mV for 10 ms.

Compounds were added to the cells in a cumulative fashion during and for each cell, tetrodotoxin (TTX, 1 μM) was applied after the compound applications of μ-conotoxin KIIIA or mimetics/controls to act as a positive control as Naᵥ1.4 and Naᵥ1.6 are TTX sensitive.

A baseline correction was applied to the patch clamp data generated on the QPatch. This data was exported and analyzed using GraphPad Prism 8.0 for Windows (San Diego, California). Nonlinear regression was performed and a 4-parameter logistic fit was used with the bottom of the curve constrained to 0. This fit was used to calculate the IC₅₀ and Hill Coefficient values. For minor isomer 2 at Naᵥ1.6 the maximal block was not sufficient to generate IC₅₀ or Hill Coefficient values.

### 2.5 NMR assignment of stapled peptide alkene isomers 3, 4 and 6

Samples were dissolved at between 0.2 and 1 mM in H₂O with 5% D₂O and 250 p.p.m. TSP as a reference and 600 μL placed in a 5 mm NMR tube (Wilmad 535-PP-7). All experiments were recorded using a Bruker AVIII HD spectrometer operating at 599.87 MHz for ³¹H using a TCI cryoprobe. For assignment and structure determination, ¹H-¹H TOCSY, DQF-COSY, ¹H-¹³C HSQC and ¹H-¹¹B NOESY spectra with zero quantum suppression were recorded at 278 K. The NOESY data for structure determination were collected at three different mixing times (80, 200, 300 ms). From 100 structures calculated in the final iteration, the 20 lowest energy were refined in explicit water and the structure with the lowest overall energy used in further analysis.

### 3 RESULTS AND DISCUSSION

#### 3.1 Design and synthesis of peptidomimetics

Synthesis of native μ-KIIIA and stapled KIIIA-mimetics was achieved with ChemMatrix Rink amide resin using standard Fmoc-based solid
phase peptide synthesis (SPPS). The native linear μ-KIIIA peptide was cleaved from the resin and purified before being oxidized to yield a complex mixture (Scheme 1) from which the major isomer (1, eluting first [see insert in scheme]) and the minor isomer (2, eluting second) could be isolated. Disulfide connectivities of these two isomers have previously been reported. [29] The two native isomers were tested to ensure biological efficacy (vide infra).

As the published NMR structure of the native μ-KIIIA (isomer 1) is α-helical [29] we replaced the disulfide bond network with synthetic staples which have been shown previously to stabilize α-helical structures by bridging either one (i to i + 4) or two helical turns (i to i + 7) (Figure 1). [30] A previous study on μ-KIIIA found six residues to be important for biological activity: K7, W8, R10, D11, H12 and R14. [31] We based the produced mimetics on this truncated 6-residue core incorporating (Scheme 2A) being isolated, whereas and (Scheme 2B) to yield the 1,5-disubstituted 1,2,3-triazole (7). We also applied Ru(l)-catalyzed azide-alkyne cycloaddition (RuAAC) on resin (Scheme 2E) to yield the 1,5-disubstituted 1,2,3-triazole i + 4 stapled mimetic 8.

To compare our mimetics with the most potent μ-KIIIA mimic reported by Khoo et al., [23] we synthesized the lactam analogue 9 using a modified method, as the authors had used Boc-chemistry. To make a direct comparison with the reported mimic, we prepared this analogue without α-methyl amino acid substitution. We employed an orthogonal protection strategy, using 2-phenyl isopropyl ester (O-2-PhPr) protected Asp3, and 4-methoxytrityl (Mmt) protected Lys7, that were simultaneously removed in 2% trifluoroacetic acid (TFA) in dichloromethane. Macro-lactamisation was achieved on resin (Scheme 2F) to provide the stapled peptide, which was cleaved and purified by RP-HPLC.

Finally, we synthesized the three linear peptide controls 10, 11 and 12 (Figure 1). Peptides 10 and 11 are alanine analogues of the i,
i + 4 and i + 7 stapled peptides, respectively. Peptide 12 incorporates Alb residues to investigate the effect of the α-methyl group in the i, i + 7 peptidomimetics.

3.2 | Patch clamp evaluation of μ-KIII A-mimetics in hNa\textsubscript{v,1.4} and hNa\textsubscript{v,1.6} expressing HEK cells

The two native isomers (1-2), the stapled mimetics (3-9) and the controls (10-12) were evaluated in a patch-clamp assay using human embryonic kidney (HEK) cells expressing the human Na\textsubscript{v,1.4} (hNa\textsubscript{v,1.4}) or Na\textsubscript{v,1.6} (hNa\textsubscript{v,1.6}) channels (Figure 2).

The major native isomer 1 gave an IC\textsubscript{50} of 200 nM against hNa\textsubscript{v,1.4} and IC\textsubscript{50} of 600 nM against hNa\textsubscript{v,1.6}, whereas the minor isomer 2 was ~30 times (hNa\textsubscript{v,1.4}) and markedly (hNa\textsubscript{v,1.6}) less active than the major isomer. Interestingly, the three i + 7 mimetics 3-5 were the only mimetics to show inhibition of the two channels; in hNa\textsubscript{v,1.4} the trans 3 and reduced 5 staples showed almost equipotent inhibition, with the trans staple 3 having a slightly better IC\textsubscript{50} of 36.4 μM, compared to 38.9 μM for the reduced staple 5 (Table 1). The cis stapled mimic 4 was 3.9-fold less active than the trans staple 3 with an IC\textsubscript{50} of 140.9 μM. Against hNa\textsubscript{v,1.6} channels, however, only the reduced staple 5 showed low micromolar inhibition with an IC\textsubscript{50} of 76.23 μM, whereas the trans 3 and cis 4 staples were 1.4- and 3.8-fold less potent, respectively. None of the i, i + 4 mimetics 6-9 or the controls 10-12 showed any inhibition in either channel type (Figure S1 in the SI), indicating that the longer i + 7 staples seem to be required to provide a bioactive conformation.

3.3 | Circular dichroism of the μ-KIII A-mimetics

The circular dichroism (CD) spectra of each of the peptides in water were acquired to evaluate the degree of α-helicity induced by the different conformational constraints to that of the linear controls (Figure 3). All peptides were run at two concentrations (50 and 100 μM) to ensure the results were independent of concentration (data for 100 μM shown in Figure 3), and then corrected to mean residue ellipticity (MRE; see SI).

The CD data for the three i + 7 HCS mimetics (3-5) and the two linear i + 7 controls (11 and 12) are presented in Figure 3A. CD data for the four i + 4 stapled mimetics (6-9) and the linear i + 4 control (10) are shown in Figure 3B. We examined the α-helicity of the compounds based on the three extrema; the maxima at 190 nm and the two minima at 208 nm and 222 nm, which are known to be indicators of α-helical structure. For the i + 7 staples there was a striking pattern; the trans-alkene hydrocarbon mimic 3 (dark green) showed most α-helical character, followed by the reduced hydrocarbon-staple 5 (purple), with the cis-alkene hydrocarbon mimic 4 (dark blue) showing the least α-helical character. Of the i + 4 mimetics, the cis-alkene hydrocarbon mimic 6 (red), showed the most α-helical character, followed by the lactam-stapled mimetic 9 (light green). Interestingly, the 1,5-triazole stapled-mimetic 8 (light blue), showed good α-helical character, whereas the CD curve from the 1,4-triazole stapled-mimetic 7 (orange) indicated a random coil conformation. We have previously shown that a 1,5-triazole bridge is a better disulfide surrogate than the 1,4-triazole analogue. The current data suggests that a 1,5-triazole bridge provides a similar type of constraint as a cis-i + 4 hydrocarbon alkene staple and can effectively promote α-helical
character in a peptide. The linear peptide controls 10, 11 and 12 all show significantly less α-helical character than the stapled peptidomimetics.

The longer i + j = 7 staples seem to be required to provide a bioactive conformation and biological activity against hNa_{1,4} and hNa_{1,6}. However, there appears to be little correlation between the CD peptide helicity of the i + j = 4 constrained analogues and bioactivity. Thus, we decided to explore the secondary structure of the three most α-helical compounds (3, 5 and 6) according to CD using solution-phase NMR spectroscopy.

### 3.4 Conformational evaluation of mimetics 3 and 5 by NMR

NMR spectroscopy has played a key role in providing the structures of peptide toxins and facilitating structure activity relationship studies.[29] The use of NMR/X-ray structural methods provides an accurate atomistic description of a peptide structure; however they require the computational description of nonnatural linkages and
only NMR spectroscopy can provide information concerning solution structural dynamics. Therefore, for the most active all-hydrocarbon mimetics 3 and 5 we produced modified CNS topology/parameter descriptions (Figures S8 and S9), which in conjunction with NOE-derived distance restraints, were used to compute solution structures of these mimetics. Near complete resonance assignment was achieved despite the degeneracy of the alkane linker signals and the calculated structures were well converged with good agreement with the experimental data (Tables S1 and S2).

Peptidomimetics 3 and 5 are α-helical throughout the sequence bounded by the linker residues and are structurally similar to each other (0.52 Å backbone RMSD between the representative structures), consistent with the similar bioactivity observed for each mimetic against hNaV1.4 (Figure 4A). Comparison of each of the mimetics with the structure of the native toxin (Figure 4B,C) shows that the amino acids...
essential for bioactivity, namely K7, W8, R10, and D11, are positioned similarly. The side chain of W8 in both mimetics is comparatively unconstrained compared to the native toxin, and more so in mimetic 3, which may explain the difference in activity at the two different Na<sub>V</sub>-channels. In the native μ-KIII A, W8 interacts with the Cys-rich core, while in the mimetics the linkers are too distant to contact it.
A feature of the native structure is that, beyond H12, the helix unwinds to make the turn into the Cys-rich core, whereas the mimetics remain helical throughout, and thus do not effectively mimic the C-terminal end of the native structure. The deviation is clearest in the backbone conformation of R14, which in the mimetic structures is firmly within a helical turn controlled by the adjacent \( \alpha \)-x-disubstituted nonnatural amino acid, whereas in the native structure the helix is essentially uncoiled (Figure 4C). Replacement of the terminal Arg has previously been shown to greatly reduce the binding affinity of the helix is essentially uncoiled (Figure 4C). Replacement of the terminal Arg has previously been shown to greatly reduce the binding affinity of the mimetics. The NMR spectra of the cis-stapled mimetic 6 does not stabilize the structure in a single conformation. As CD and NMR are sensitive to structural changes on different timescales\(^{[42]}\) this may lead to seemingly contradictory results as observed for mimetic 6. In the context of peptidomimetics, care must be taken not to over interpret CD spectroscopic data. A helical curve does not necessarily mean a rigid, highly constrained helical structure. NMR analysis of peptides that are conformationally dynamic yet spend a significant proportion of their time in a helical conformation can give rise to NOE cross peaks that suggest only a helical structure. It is therefore important to evaluate secondary structure using both methods. However, we found that NMR provides more useful structural information that can be used to rationalize biological data.

4 | CONCLUSIONS

The voltage-gated sodium channels comprise a variety of related proteins with distinct physiological and pharmacological properties with specific subtypes localized to brain regions, skeletal muscle, peripheral neurons, and heart. Local anesthetic drugs work by blocking these channels and interrupting the flow of action potentials in neurons carrying nociceptive (and other sensory) information from the periphery to the central nervous system. The \( \mu \)-conotoxins present an opportunity to create safe, selective anesthetics but in their native form these peptides are unlikely to become lead drug compounds due to their unfavorable physicochemical properties.

In this work, we produced simplified structures based on \( \mu \)-KIIIA by removing the complex disulfide-bonding network and replacing it with chemical staple conformational constraints. We synthesized seven stapled mimetics using a variety of chemistries and compared them to the native \( \mu \)-KIIIA isomers as well as three nonstapled control compounds. Only compounds containing the \( ii + 7 \) staples (3-5) gave low micromolar inhibition of the two tested human sodium channels, \( \text{Na}_v1.4 \), from skeletal muscle and \( \text{Na}_v1.6 \) from the CNS. Interestingly, the reduced hydrocarbon-staple 5 could inhibit both h\( \text{Na}_v1.4 \) and h\( \text{Na}_v1.6 \) channels equally well, whereas the trans-staple 3 showed good inhibition of \( \text{Na}_v1.4 \) but not \( \text{Na}_v1.6 \), indicating this compound could be used to selectively target one channel. The cis-staple 4 was also active, but significantly less than the other two mimetics, having a less favorable interaction with the binding sites. None of the other mimetics or controls were active, indicating that the structure of these compounds is key to biological activity. This was surprising, as Khoo et al.\(^{[39]} \) had previously shown lactam-staple 9 to be active in rodent \( \text{Na}_v1.4 \) and \( \text{Na}_v1.6 \) channels. This species difference in receptor pharmacology highlights the importance of testing compounds using human VGSC assays in drug discovery programmes.

As the native \( \mu \)-KIIIA adopts an \( \alpha \)-helical conformation due to the complex disulfide-bonding network, we explored the secondary structure of our mimetics using CD. The data indicate little correlation between the CD peptide helicity and biological activity. Thus, we decided to examine the two most active mimetics 3 and 5 as well as the most helical \( ii + 4 \) staple (the cis-staple 6), (according to CD), using solution NMR spectroscopy.

The two active mimetics 3 and 5 have similar structures to the native \( \mu \)-KIIIA helix, but at the C-termini the stapled peptides' backbones continue the helical trajectory where the native structure unwinds. As a result, the positioning of the backbone atoms of the linker residue that substitutes S13 and of R14 deviate significantly from those in the native structure, possibly explaining the lower activity of the mimetics. The NMR spectra of the cis-stapled mimetic 6 indicate that the compound was not stabilized in a well-defined conformation.

With an understanding of the structure-activity relationship of these peptidomimetics, our future investigations will focus on developing potent, selective human \( \text{Na}_v \)-channel blockers.

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CONFLICT OF INTERESTS

There are no conflicts to declare.

DATA AVAILABILITY STATEMENT

The Supporting Information is available free of charge on the Wiley publications website at https://doi.org/10.1002/pep2.24203. Experimental procedures and characterization data for all compounds, NMR spectra, Patch-clamp assay and analytical HPLC traces for final compounds.

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