Structural and functional insights into the inhibition of human voltage-gated sodium channels by μ-conotoxin KIIIA disulfide isomers

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Hue N. T. Tran, Kirsten L. McMahon, Jennifer R. Deuis, Irina Vetter, and Christina I. Schroeder

From the Institute for Molecular Bioscience, The University of Queensland, St Lucia, Queensland, Australia; School of Pharmacy, The University of Queensland, Woolloongabba, Queensland, Australia; Center for Cancer Research, National Cancer Institute, National Institutes of Health, Frederick, Maryland, USA

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μ-Conotoxins are components of cone snail venom, well-known for their analgesic activity through potent inhibition of voltage-gated sodium channel (NaV) subtypes, including NaV1.7. These small, disulfide-rich peptides are typically stabilized by three disulfide bonds arranged in a ‘native’ CysI-CysIV, CysII-CysV, CysIII-CysVI pattern of disulfide connectivity. However, μ-conotoxin KIIIA, the smallest and most studied μ-conotoxin with inhibitory activity at NaV1.7, forms two distinct disulfide bond isomers during thermodynamic oxidative folding, including Isomer 1 (CysI-CysV, CysII-CysIV, CysIII-CysVI) and Isomer 2 (CysI-CysVI, CysII-CysIV, CysIII-CysV), but not the native μ-conotoxin arrangement. To date, there has been no study on the structure and activity of KIIIA comprising the native μ-conotoxin disulfide bond arrangement. Here, we evaluated the synthesis, potency, sodium channel subtype selectivity, and 3D structure of the three isomers of KIIIA. Using a regioselective disulfide bond-forming strategy, we synthetically produced the three μ-conotoxin KIIIA isomers displaying distinct bioactivity and NaV subtype selectivity across human NaV channel subtypes 1.2, 1.4, and 1.7. We show that Isomer 1 inhibits NaV subtypes with a rank order of potency of NaV1.4 > 1.2 > 1.7 and Isomer 2 in the order of NaV1.4 ≈ 1.2 > 1.7, while the native isomer inhibited NaV1.4 > 1.7 ≈ 1.2. The three KIIIA isomers were further evaluated by NMR solution structure analysis and molecular docking with hNaV1.2. Our study highlights the importance of investigating alternate disulfide isomers, as disulfide connectivity affects not only the overall structure of the peptides but also the potency and subtype selectivity of μ-conotoxins targeting therapeutically relevant NaV subtypes.

Pain, in particular neuropathic and inflammatory, is of major medical concern worldwide. Voltage-gated sodium channels (NaVs) have been proven to play an essential role in many different pain states in animal and human models (1–7). There are nine sodium channel subtypes (NaV1.1–NaV1.9) currently described, all sharing a common overall structural motif (8). Most neurons express multiple sodium channel isoforms with NaV1.1 (9), NaV1.2 (10), NaV1.3 (11, 12) and NaV1.6 (13) being expressed in the central nervous system, while NaV1.4 and NaV1.5 are expressed in skeletal (14) and cardiac muscles (15), respectively. NaV1.7 (16), NaV1.8 (17), and NaV1.9 (3) subtypes are preferentially expressed in the peripheral nervous system. These peripheral sodium channels have been genetically validated to be modulating neuronal excitability associated with different types of pain, including nociception (17, 18), neuropathic pain (3, 5, 18, 19) and acute inflammation (8, 16, 20, 21). Thus, peripheral NaV subtypes, such as NaV1.7, are potential targets for the development of novel analgesics.

Conotoxins from the venom of marine cone snails are considered prospective drug leads because of their potency, NaV subtype selectivity, and analgesic efficacy in animal and human trials (1, 22). μ-Conotoxins are 14 to 26 amino acid residue peptides that share a specific cysteine framework of CCX₅CX₄CX₄CC (23–25). The μ-conotoxin super family comprises five branches, termed M-1 to M-5, based on the number of residues in the third cysteine loop between the fourth and fifth cysteine residues (25). With a framework including six cysteine residues, the μ-conotoxins can theoretically form 15 different disulfide bond isomers. However, μ-conotoxins isolated directly from cone snail venom are typically found to adopt a CysI-CysIV, CysII-CysV, CysIII-CysVI conformation, which has accordingly been accepted as the ‘native’ fold of the μ-conotoxin family (25). However, if isolated peptide material is not available for coelution with synthetic material, for example, when peptide sequences are identified from cDNA venom duct libraries, using proteomics or transcriptomics, or when the peptide is not present, or expressed at very low levels in the venom, it can be challenging to ascertain the native fold of the peptide. Accordingly, some recent reports have shown that it is not always the ‘native’ conotoxin peptide fold that is the more potent biologically active peptide, and conotoxins with non-native disulfide bond connectivity can outperform the presumed ‘native’ counterpart (26–28). For example, synthetic μ-PIIIA, a μ-conotoxin identified from the cDNA of the venom duct of the cone Conus purpurascens (26), formed three active isomers under...
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thermodynamic oxidative channels with the non-native isomer of PIIIA (CysI-CysV, CysII-CysVI, CysIII-CysIV) being slightly more potent than the PIIIA isomer comprising the native μ-conotoxin disulfide-bond arrangement (27). A similar peculiarity has been observed within the α-conotoxin family, where a non-native disulfide bond isomer of the 15-residue α-conotoxin AuB (comprising two disulfide bonds) was 10-fold more potent than the native fold of the peptide (28). Discoveries such as these emphasize the importance, but also the potential, of further investigation into the activity of different disulfide bond connectivities of these small, highly disulfide-rich peptides and how different disulfide connectivities of the same peptide can influence not only potency but also receptor subtype selectivity. Herein, we focus our investigation on three different disulfide-bond isomers of μ-KIIIA, one of the most extensively investigated μ-conotoxins targeting NaV1.7 (29, 30).

KIIIA is a 16-residue μ-conotoxin, identified from a cDNA venom duct library of Conus kinoshitai that acts by blocking the pore of NaVs (20, 31–33). KIIIA is the smallest μ-conotoxin identified to date (34), comprising only one residue in loop 1 of the peptide, compared to 4 to 6 for other μ-conotoxins (Table 1). Like other μ-conotoxins in the family, KIIIA has been reported to most potently inhibit rat NaV1.2 and rat NaV1.4 (29, 30, 35). However, unlike most μ-conotoxins, KIIIA also inhibits the peripheral analgesic target NaV1.7 with nanomolar affinity (29, 30). Upon initial synthetic production, KIIIA was reported to form one major isomer during thermodynamic folding (31), which was assumed to comprise the native μ-conotoxin disulfide connectivity (CysI-CysIV, CysII-CysV, CysIII-CysVI). A later study by Khoo et al. (35) reported two isomers during thermodynamic folding, and by carrying out direct mass spectrometric collision-induced dissociation fragmentation, it was identified that in fact neither of the two isomers observed during thermodynamic folding were KIIIA comprising the native μ-conotoxin fold (35). Instead, the two synthetically produced KIIIA isomers comprised CysI-CysV, CysII-CysIV, CysIII-CysVI connectivity (thermodynamic Isomer 1, referred to as Isomer 1-T) as the primary peak and CysI-CysVI, CysII-CysIV, CysIII-CysV connectivity (thermodynamic Isomer 2, referred to as Isomer 2-T) as the minor peak, with the minor isomer reported to be five times less potent than Isomer 1 against rNaV1.2 (35). Indeed, as of yet, there has been no studies on KIIIA comprising the native μ-conotoxin disulfide connectivity of CysI-CysIV, CysII-CysV, CysIII-CysVI.

KIIIA and other small μ-conotoxins have been shown to exhibit analgesic properties in mouse inflammatory pain assays (20, 29, 36) and it is believed that the size and framework of μ-conotoxins, coupled with their high affinity and unique NaV channels selectivity, make them prospective therapeutic agents suitable for the development of novel analgesic drugs (22, 25, 29, 37–44). To better understand the structure and activity of KIIIA isomers and the influence of different disulfide connectivity on NaV affinity and subtype selectivity, we chemically produced three isomers of KIIIA including Native, Isomer 1, and Isomer 2 using a regioselective oxidation strategy. Our results revealed differences in NaV subtype selectivity and potency between the three disulfide isomers that could partially, albeit not wholly, be explained by structural studies and molecular modeling.

Results

Synthesis

KIIIA and analogs were assembled using 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide chemistry. Unprotected KIIIA was thermodynamically oxidized in 0.1 M NH₄HCO₃, pH 8, 100 eq GSH, and 10 eq GSSG producing two distinct isomers (Isomer 1-T and Isomer 2-T, where T stands for thermodynamic) (Fig. 1A) that showed similar relative retention times and peak height ratios as those observed by Khoo et al. (35) The two thermodynamically folded isomers, Isomer 1-T and Isomer 2-T, were readily isolated by reverse-phase HPLC (RP-HPLC). To ensure correct disulfide bond-formation, regioselective synthesis of each isomer producing the desired connectivity was carried out (Isomer 1-R and Isomer 2-R, where R stands for regioselective). However,

Table 1

| μ-conotoxins | Sequences | References |
|--------------|-----------|------------|
| KIIIA        | CC——N——CSKKWCARDH5RCC* | (31) |
| KIIIB        | NGCC——CSKKWCARDH5RCC* | (31, 35) |
| SIIIA        | ZNCC——NG——GCSKKWCARDH5RCC | (31, 92, 93) |
| SIIIB        | ZNCC——NG——GCSKKWCARDH5RCC | (93) |
| SmIIIA       | ZRCC——NGRRGCSSRWCARDH5RCC* | (76) |
| GIIIA        | RDCG——TOOKK——CDDQCKOD——KCCA* | (94) |
| GIIHB        | RDCG——TOOKK——CDDRCKOM——KCCA* | (94) |
| GIIIC        | RDCG——TOOKK——CDDRCKOL——KCCA* | (94) |
| TIIIA        | RHGC——KGOKGC——CSSRWCROOH——CC | (93) |
| PIIB         | RLCG——GFOKCS——ERSRQCKOH——CC | (26, 27) |
| BuIIIA       | VTRDCG——K——NGKRGSC——RWGARDH5RCC | (95) |
| BuIIIB       | VGERCC——K——NGKRGSC——RWWCSSH5RCC | (95) |
| BuIIIC       | IVDRCNKGKRGSRW——C——RWWCSSH5RCC | (95) |
| CIIB         | GRCG——EGPNGCSSRWCARDH5RCC | (96) |
| CaIIIA       | GRCG——DPVNASCSSKWCARDH5RCC | (96) |
| CnIIIB       | ZGCG——GEPNLCFTWRCCRNRACCCRCQQ* | (96) |
| MiIIIA       | ZGCG——NPNGCSSRWCARDH5RCC | (96) |
| SxIIIC       | RGCG——NGRGGC——CSSRWCARDH5RCC | (53) |

* C-terminal amidation; Z, pyroglutamate; O, hydroxyproline; KIIIA highlighted in gray; Cys residues highlighted in yellow.
directing the disulfide connectivity of KIIIA proved challenging (Table S1 and Figs. S1–S3) and after trialing a variety of regioselective protecting group strategies including 4-methyltrityl/dimethylphosphinyl/S-acetamidomethyl (Acm), S-triphenylmethyl (Trt)/p-methoxybenzyl (Mob)/Acm, Trt/Acm/4-methylbenzyl (Mebzl), and Trt/Acm/Mob, we found that only the combination of using Cys with protecting groups Trt to form the first disulfide bond, Acm to form the second disulfide bond followed by 4,4'-dimethylsulfonylbenzhydryl (Msbh) to form the final third disulfide bond, successfully produced Native KIIIA (Table S1 and Fig. 2). Using this orthogonal cysteine-protecting group strategy, we were subsequently able to produce Isomer 1-R (CysI-CysV, CysII-CysIV, CysIII-CysVI), Isomer 2-R (CysI-CysVI, CysII-CysIV, CysIII-CysV), and Native (CysI-CysIV, CysII-CysV, CysIII-CysVI) KIIIA with ~10% yield from crude peptides (Table S2 and Fig. S4).

**Assignment of disulfide bond connectivity by coelution**

Linear KIIIA formed two isomers during thermodynamic oxidation, as shown by analytical HPLC (Fig. 1A). The major peak, Isomer 1-T, coeluted with regioselectively synthesized

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**Figure 1.** Analytical RP-HPLC traces showing disulfide bond isomers of KIIIA obtained through thermodynamic oxidation and isomers obtained through step-wise directed folding conditions. A, Isomer 1-T and Isomer 2-T formed in thermodynamic condition (black), directed Isomer 1-R (blue), Isomer 2-R (green), and Native (red) conformation. B, Isomer 1-T (black) coeluted with Isomer 1-R (CysI-CysV, CysII-CysIV, CysIII-CysVI) (blue), Isomer 2-R (CysI-CysVI, CysII-CysIV, CysIII-CysV) (green) and Native (CysI-CysIV, CysII-CysV, CysIII-CysVI) isomer (red) and was ultimately assigned as Isomer 2-R based on NMR. RP-HPLC, reverse-phase HPLC.

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**Figure 2.** Regioselective synthesis of Native KIIIA (CysI-CysIV, CysII-CysV, CysIII-CysVI), forming disulfide bonds in the order of CysIII-CysVI, CysII-CysV, and CysI-CysIV. A, synthetic regioselective oxidation scheme showing reaction conditions for each step. B, analytical RP-HPLC traces corresponding to the product obtained by each step of the synthesis including the linear starting peptide, following the formation of first, second, and third disulfide bond. C, observed mass fragmentation corresponding to each folding step acquired by LC-MS. * denotes peptides of interest. RP-HPLC, reverse-phase HPLC.
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Isomer 1-R (CysI-CysV, CysII-CysIV, CysIII-CysVI) KIIIA, confirming the results obtained by RP-HPLC and NMR (Figs. 1B and S5). In addition, the secondary Hu chemical shifts of Isomer 1-R superimposed well with chemical shifts from the structure of KIIIA published by Khoo et al. (Fig. S5E) (34), confirming that Isomer 1-T corresponds to the previously described structure with connectivity of CysI-CysV, CysII-CysIV, CysIII-Cys VI. In our hands, the minor peak obtained from thermodynamic folding, Isomer 2-T, coeluted with both Isomer 2-R (CysI-CysVI, CysII-CysIV, CysIII-CysV) and Native KIIIA (CysI-CysV, CysII-CysIV, CysIII-CysVI) produced regioselectively (Fig. 1C). However, using NMR, we could delineate the differences in their structures via 1D 1H NMR spectra and secondary Hu chemical shifts (Fig. S5). 1D 1H NMR spectra showed good dispersion of peaks in the order of Isomer 1 > Isomer 2 > Native. Isomer 1 was found to produce the highest quality spectrum with sharp peaks. Compared to Isomer 2, the Native NMR spectra were not of similar sharpness and dispersion (Fig. S5A).

Activity and selectivity of KIIIA isomers at human NaV channels

Evaluation of inhibitory activity and subtype selectivity of the three KIIIA isomers across hNaV1.2, hNaV1.4, and hNaV1.7 by automated whole-cell patch-clamp electrophysiology (Fig. 3) provided insights into differences in activity displayed by the three different peptides. All three isomers inhibited hNaV1.2, hNaV1.4, and hNaV1.7 with distinct potency and subtype selectivity (Fig. 3). Across hNaV channels tested, Isomer 1 was the most potent inhibitor overall, followed by Native KIIIA and Isomer 2 (Table 2). However, the selectivity profiles of the three isomers showed preferential inhibition of NaV1.4 over NaV1.2 and NaV1.7 by Isomer 1 (NaV1.4 > NaV1.2 > NaV1.7), the Native isomer displayed selectivity for NaV1.4 > NaV1.7 = NaV1.2, and Isomer 2 was approximately equipotent at NaV1.4 and NaV1.2 displaying diminished activity at NaV1.7 (NaV1.4 = NaV1.2 > NaV1.7).

Compared to Isomer 1, KIIIA Isomer 2 was more than 10-fold less active on NaV1.2 (p < 0.0002), 31-fold less potent on NaV1.4 (p < 0.0002), and 13-fold less active on hNaV1.7 (p < 0.0001) (Table 2 and Fig. 3). On the other hand, Native KIIIA was more than 7-fold less active on NaV1.2 and 10-fold less potent on NaV1.4 while only being 2-fold less active on hNaV1.7 compared to Isomer 1 (Table 2 and Fig. 3). These results demonstrate that disulfide connectivity can affect both potency and subtype selectivity and that the presumed native fold may not always be the most potent isoform at nonpreyspecific pharmacological targets, or display the most desirable selectivity profile at human NaV channels.

3D NMR structure of KIIIA isomers

To better understand factors driving the distinct potency and selectivity of the three KIIIA isomers, we investigated the 3D structures of Isomer 1, Isomer 2, and Native KIIIA (Fig. 4, A–C) using homonuclear 1H NMR. TOCSY and NOESY spectra were used to assign individual spin systems and the sequential walk (45) in Ccpnmr (46). Intra-, inter-, and long-range NOEs were assigned for individual peptides, and an initial 20 structures were calculated using the AUTO function in Cyana followed by refinement in a watershell in CNS (47). Structural statistics for the three isomers were evaluated using Molmol (48) and Molprobity (49) and a family of 20 structures with the lowest energy and best Molprobity scores were chosen to represent each of the peptides (Table S3). The solution structures of Native KIIIA and KIIIA Isomer 2 have been submitted to the Protein Data Bank (KIIIA Native PDB ID: 7SAV and KIIIA Isomer 2 PDB ID: 7SAW) and the Bio Magnetic Resonance Bank (KIIIA Native BMRB: 30953 and KIIIA Isomer 2 BMRB: 30954). All three isomers maintained the characteristic α-helix in the central loop of the peptide. Isomer 1 exhibited a very compact structure for μ-conotoxins, with a typical α-helical turn from Ser6 to Ser13 (34, 35). Native KIIIA also produced a very tight structure though its α-helix was observed to be shorter compared to Isomer 1, stretching from Ser6 to His12 (Fig. 4). Across the α-helical segment, the 3D structures of Isomer 1 and Native KIIIA were well structured with RMSD’s of 0.35 ± 0.1 Å and 0.4 ± 0.13 Å, respectively. The main difference between the two structures is noted in the N- and C-termini, which are oriented in different directions due to the constraint brought about by the cystine connectivity. Isomer 2 also displays an α-helical turn stretching from residue Ser6 to His12, though this helix (RMSD 0.54 ± 0.23 Å) exhibits more flexibility than Isomer 1 or Native, corresponding with fewer hydrogen bonds being observed across this stretch of amino acids for Isomer 2 (Table S3). Sidechain orientations resides which are responsible for interaction with the channels (36), such as Arg14, Ser13, His12, Asp11, Arg10 in Isomer 1 and Native KIIIA are similar, though these sidechains are orientated differently in Isomer 2 (Fig. 4, A–C). Overall, these structural differences, in particular, the shorter and more flexible helical structure of Isomer 2 (four hydrogen bonds in region Ser5–His12) compared to the compact α-helical structure of Isomer 1 and Native KIIIA (six hydrogen bonds each in the Ser6–His12 region), and the resultant changes in structural flexibility and sidechain positioning, could explain the relative decrease in potency observed for Isomer 2 in this study.

KIIIA isomers docking with hNaV1.2

To begin to understand how these relatively minor structural differences might contribute to the observed functional differences, we further investigated the interactions of Isomer 1, Isomer 2, and Native KIIIA with hNaV1.2 using the structure of hNaV1.2 solved in combination with KIIIA, displaying a CysI-CysV, CysII-CysIV, CysIII-CysVI disulfide bond connectivity (Isomer 1-T), published by Pan et al. (PDB 6J8E) (50). The three isomers from this study were docked onto hNaV1.2 containing a β2-subunit using Autodock VINA (51) and docking models with the lowest energy were chosen for further analysis. These docking studies confirmed that our Isomer 1-R and Native KIIIA (Fig. 4D), but not Isomer 2 (Fig. 4E), superimposed well with KIIIA in the hNaV1.2 cryo-EM complex. Possible interactions between the residues of KIIIA
Figure 3. Pharmacology of KIIIA isomers at hNaV channels. A, representative current trace before and after the addition of Isomer 1 (blue), Isomer 2 (green), and Native KIIIA (red) at hNaV1.2, hNaV1.4, and hNaV1.7. B-D, concentration-response curve showing inhibitory activity of KIIIA isomers at hNav1.2, hNaV1.4, and hNaV1.7 respectively, the data are presented as mean ± SEM. E, selectivity profile of KIIIA Isomer, Isomer 2, and Native acquired using automated whole-cell patch-clamp electrophysiology on HEK293 cells overexpressing hNaV1.2, hNaV1.4, or hNaV1.7 in combination with β1-subunits. The data are presented as mean ± SD, with n = 5 cells per data point. 2-way ANOVA significance * p value < 0.0332, ** p value < 0.0021, *** p value < 0.0002, **** p value < 0.0001. NaV, voltage-gated sodium channel.
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Table 2

Activity of KIIIA isomers on hNaV1.2, hNaV1.4, and hNaV1.7 evaluated using automated electrophysiology

| Sodium channel subtypes | Isomer 1 | Isomer 2 | Native KIIIA |
|-------------------------|----------|----------|--------------|
| hNaV1.2                 | 124 ± 34 nM | 1371 ± 403 nM | 875 ± 129 nM |
| hNaV1.4                 | 65 ± 15 nM  | 2051 ± 482 nM | 472 ± 94 nM  |
| hNaV1.7                 | 413 ± 71 nM | 5388 ± 547 nM | 887 ± 295 nM |

The data are presented as mean ± SD, with n = 5 cells per data point.

Isomers and hNaV1.2 were identified by PDBsum (52) and are summarized in Figure 4F and Fig. S6. We first compared interactions in the Lys7–Ser13 region of docked Isomer 1 and the cryo-EM complex (50). There were multiple similarities between the two complexes, that is, Lys7 of KIIIA forms a hydrogen bond with Glu945 on hNaV1.2, Trp8 interacts via nonbonded interactions with Tyr363, Arg10 interacts with Arg922 and Asp1426, Asp11 with Arg922, His12 with Ile914, Ser915, and Asp916, and Ser13 with Asn916, validating our docking study. Furthermore, when comparing the interactions of Isomer 1, Isomer 2, and the Native conformation of KIIIA with hNaV1.2, we observed that Isomer 1 and the Native conformation KIIIA shared many interactions (Figs. 4F and S6). In contrast, Isomer 2 only shared two interactions with Isomer 1 and Native KIIIA, including His12 and Ser13 with Asn916. These differences and similarities between Native and Isomer 1/Isomer 2 could explain why Native KIIIA is more potent than Isomer 2 but less potent than Isomer 1 across the subtypes evaluated in this study. We also compared the interactions between residues in the termini of Isomer 1 and the Native KIIIA conformation with hNaV1.2. Compared to the Native conformation of KIIIA, we noticed that Isomer 1 had two and three more interactions with the channel at the N- and C-terminus, respectively. These observations could explain why Isomer 1 is slightly more active at hNaV1.2 than Native KIIIA. The results from these docking studies will need to be experimentally validated through structure-activity relationship studies of the peptide in combination with corresponding channel mutants and docking studies with NaV1.4 and NaV1.7 structures which may identify other important interactions.

Discussion

μ-Conotoxins, in particular KIIIA, have long been of interest as leads for novel analgesics due to their small size, chemical stability, and pharmacological profile (20, 29, 36). Like most μ-conotoxins, KIIIA displays activity at the neuronal NaV1.2 and the skeletal muscle isoform NaV1.4, which could be associated with adverse effects. However, KIIIA is one of only a few known μ-conotoxins exhibiting nanomolar potency at hNaV1.7 (36, 50, 53–55). The KIIIA sequence was initially identified from a CDNA venom duct library from C. kinoshitai (31) and was subsequently chemically synthesized and folded thermodynamically, resulting in the formation of two disulfide bond isomers (Isomer 1; CysI-CysV, CysII-CysVI, CysIII-CysVI and Isomer 2; CysI-CysVI, CysII-CysIV, CysIII-CysV) as reported by Khoo et al. (35) However, it remains unclear which isomer(s) of KIIIA exist in C. kinoshitai venom (if it is present at all), what the ‘real native’ disulfide connectivity of the venom peptide is, and what machinery assists the snail with the folding of the peptide. Here, we report the regioselective synthesis of three KIIIA isomers, including for the first time KIIIA with the ‘native’ μ-conotoxins disulfide connectivity, their activity, selectivity, and their structure and interactions with human NaV's.

Synthesis

While the development of an extensive repertoire of Cys-protecting groups has expanded the toolbox for regioselective oxidation of complex disulfide-rich peptides (56–63), complete orthogonal production of a peptide like KIIIA, containing three disulfide bonds (37.5% overall Cys content) is still not always straightforward. Directed synthesis of up to four disulfide bonds in a cysteine-rich peptide has been accomplished by using various protecting-group schemes with either Boc (tert-butoxycarbonyl) or Fmoc chemistry (63); most commonly with combinations of Trt, Acm, Mebzl, Mob, Msbh, terbutyl, or S-terbutylthio groups (56–63). Whereas the robustness of the Trt, Acm, and Mebzl or Mob protecting groups is established, the removal of terbutyl groups often results in the formation of side products and low yields (64–66), and the removal of S-terbutylthio groups by reducing agents has been observed to be sequence dependent (56, 67).

After trialing several different Cys-protecting groups following the preferential order of disulfide bond formation previously described (68–70) (Table S1), we successfully produced the three KIIIA isomers pursued in this study by regioselective chemical synthesis. By following a process described by Dekan et al. (63), using a combination of Trt/Acm/Msbh-protecting groups, we synthesized all three desired isomers with a yield of ~10% calculated from the crude peptide. In general, regioselective oxidation methods are highly sequences dependent, meaning they may work for one peptide, but this may not always be transferable and work for other similar peptides.

Activity

μ-Conotoxins have previously been reported to mainly target NaV1.4 and NaV1.2 and only a few peptides, specifically KIIIA, SxIIIC, and CnIIIC, have been shown to target the thermapeutically relevant isoform hNaV1.7 (36, 50, 53–55, 71). In this study, we therefore focused our attention on these three sodium channel subtypes in order to investigate the activity and selectivity of Isomer 1, Isomer 2, and Native KIIIA. Previous selectivity and activity of KIIIA (Isomer 1) has been reported mainly for rat (20, 34, 72–74) and mouse NaV subtypes (29, 72), as well as some human NaV isoforms expressed in Xenopus laevis oocytes, with or without β subunits (50, 55, 72).
Figure 4. Three-dimensional structure of KIIIA isomers used in this study and docking studies with KIIIA-hNaV1.2 complex (Pan et al. (PDB 6J8E)) (50). A, KIIIA Isomer 1 (PDB ID: 2XLG) (blue). B, KIIIA Isomer 2 (green) (this study). C, KIIIA Native (red) (this study). D, docked KIIIA Isomer 1 (blue) and Native KIIIA (red) compared to cryo-EM of KIIIA (Isomer 1, teal) in complex with hNaV1.2. E, docked Isomer 2 (green) of KIIIA compared to cryo-EM of KIIIA (Isomer 1, teal) in complex with hNaV1.2. F, putative interactions between KIIIA isomers and hNaV1.2 compared to cryo-EM of KIIIA (Isomer 1) in complex with hNaV1.2 (50). NaV, voltage-gated sodium channel.
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The rank order of potency at human NaV subtypes has not previously been reported, however Khoo et al. (29, 34, 35) and Wilson et al. (30) observed that Isomer 1 of KIIIA inhibits NaVs with a rank order of rNaV1.2 > rNaV1.4 > mNaV1.7 ≥ rNaV1.1 > rNaV1.3 > rNaV1.5. Thus, the subtype selectivity preference for Isomer 1 of KIIIA across the human NaV subtypes tested in this study is broadly consistent with the activity reported by these previous works. Although rat and human NaV α-subunits are >95% identical at the level of amino acid sequence (75), species-specific activity differences have previously been reported for μ-conotoxins (29, 76, 77) and may account for some of the potency differences observed in this study compared to the literature.

Furthermore, coexpression of different β-subunits in heterologous expression systems can significantly affect affinity and efficacy of toxins, including μ-conotoxins (78, 79). Specifically, the on-rates (kon) of μ-conotoxins at several NaV channel isoforms can be increased in the presence of β1 and β3 subunits, while they are decreased by β2 and β4 subunits (80). In contrast, all four β-subunits increase the ko of μO-conotoxin MrVIB (a gating-modifier inhibitor) at NaV1.8 channels (29). Furthermore, coexpression of β2- or β4-subunits protects tetrodotoxin-sensitive NaV1.1 to 1.7 subtypes against block by an analog of μO5-conotoxin GVIIA (81). For KIIIA, coexpression of all rNaV isoform except NaV1.5 with β1 increased the rate of inactivation of hNaV (30).

Thus, to permit direct comparison of pharmacological activities of the three KIIIA isomers, we performed automated patch-clamp electrophysiology at human NaV isoforms 1.2, 1.4, and 1.7 co-expressed in HEK cells with the β1-subunit. Consistent with the principal activity of many μ-conotoxins at NaV1.4 and NaV1.2, the KIIIA isoforms were potent across these two isoforms. However, Native KIIIA displayed an improved relative selectivity for NaV1.7 compared with Isomer 1 and Isomer 2 driven by a loss of activity at NaV1.4 and NaV1.2. As NaV subtypes are characterized by a high degree of sequence identity (hNaV1.4 cf. hNaV1.7: 60.33% identical; hNaV1.2 cf. hNaV1.7: 77.11% identical; hNaV1.2 cf. hNaV1.4: 63.02% identical), similar functional characteristics, and a typical overall structure (5), these subtle shifts in potency and selectivity, driven by relatively modest shifts in the 3D peptide structure, remain difficult to explain. Recent advances in our understanding of the structure of NaV channels, including a cryo-EM structure of KIIIA bound to human NaV1.2/β2 (50) are particularly valuable in this regard and were thus used for docking studies to visualize putative interactions between key residues of different isoforms of KIIIA with NaV channels.

NMR and docking studies

Our experimentally determined Native KIIIA structure was highly similar across the α-helix part of the peptide compared to the structure of Isomer 1 (PDB ID: 2LXG) calculated by Khoo et al. (34, 35), consistent with the high degree of similarity between these two isomers. Due to the small size of KIIIA, the different disulfide connectivities of the three isomers had minimal effect on backbone of the structure, apart from across the N- and the C-terminus of KIIIA Isomer 2 structure (this study). However, the direction of the sidechains differed greatly, thus likely contributing to the different potency and selectivity profiles we observed.

Our docking studies also confirmed several critical interactions between KIIIA isomers and NaV1.2 that were reported by previous studies (20, 36, 72, 82). Overall, the most pharmacologically similar isomers (Isomer 1 and Native KIIIA) had more interactions in common compared to Isomer 2, which was also considerably less potent across all NaV subtypes studied. However, it is worth noting that these docking studies could be associated with some limitations. Firstly, both peptide NMR and the NaV1.2/β2 cryo-EM structures may not reflect the full range of physiologically relevant conformations, which could contribute to subtle differences in activity. For example, our pharmacological activity studies were conducted in HEK293 cells coexpressing the β1-subunit, while the KIIIA-hNaV1.2 cryo-EM structure was obtained in the presence of the β2-subunit (50). Additionally, the KIIIA-NaV1.2/β2 cryo-EM structure was obtained with Biotin-(AEEA)2-KIIIA (Isomer 1) at very high peptide concentrations (25 mM) (50). Interestingly, although several interactions identified in the cryo-EM structure could explain preferential inhibition of NaV1.2 over the tetrodotoxin-resistant isoforms NaV1.5, NaV1.8, and NaV1.9, the even greater potency of KIIIA (Isomer 1) at hNaV1.4, as well as potent activity at hNaV1.7 observed by us, cannot be fully explained by this structure given that the majority of critical interacting residues are conserved across these subtypes.

We propose that the peptide N- and C-termini also contribute to differential interactions with NaV subtypes, in addition to the helical region of KIIIA that has previously been identified to hold key residues accounting for μ-conotoxin activity at NaV channels (20, 36, 72, 82). This is supported by previous work showing that both termini of KIIIA contribute to the interaction with NaV1.7 and are sensitive to alterations (54). Specifically, the amidated C-terminus has proven critical for Isomer 1 KIIIA activity at hNaV1.7, as both amino acid C-terminal extension and deamidation resulted in complete loss of activity (54). In addition, an extension of the N-terminus of Isomer 1 KIIIA by a poly-Gly tail can either increase or decrease the potency of the peptide (35, 54). Contribution of the termini to the biological activity of KIIIA would also be consistent with the high degree of structural similarity of the isomers in the α-helical region, although this remains to be explored in greater detail at the practical level.

Conclusion

We produced three different disulfide bond isomers of KIIIA using a combination of thermodynamic and step-wise regioselective protocols evaluating a range of different Cys-protecting groups and disulfide bond formation order. We found that the combination and order of Cys(Trt), Cys(Acm), and Cys(Msbh) was the only strategy employed in this study, which was able to produce the desired folded peptides. Isomer 1, Isomer 2, and Native KIIIA were distinguishable via NMR, and although all three isomers share a similar overall 3D structure, comprising an α-helical turn located from Lys7 to
His12, they displayed different potency and selectivity profiles across hNaV1.2, hNaV1.4, and hNaV1.7. Docking of the 3D NMR structures revealed a series of interactions between the different KIIIA isomers and a hNaV1.2/β2 complex that could provide an explanation for their distinct bioactivity. The docking study showed that while the α-helical turn and a series of hydrogen bonds are critical, both the N- and C-termini of KIIIA also appear to contribute to the interaction with hNaV subtypes. Further experimental validation of such interactions may lead to the rational design of potent and selective hNaV1.7 inhibitors with therapeutic potential.

**Experimental procedures**

Unless otherwise stated, all chemicals, solvents, and reagents were purchased from Sigma Aldrich (Sigma Aldrich). Amino acids were purchased from Iris Biotech GmbH.

**Peptide synthesis**

Peptides were manually assembled on Fmoc-Rink amide polystyrene resins on a 0.125 mmol scale (RAPP Polymer, 0.69 mmol/g). 9-fluorenylmethoxycarbonyl-protected amino acid couplings were performed in dimethylformamide using 4 eq of amino acid/0.5 M HCTU (O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)/N,N-diisopropylethylamine (1:1:1) relative to resin substitution (2 × 10 min). 9-fluorenylmethoxycarbonyl removal was accomplished by treatment with 30% piperidine/dimethylformamide (2 × 2 min).

Cleavage from the resin and simultaneous removal of sidechain-protecting groups was accomplished by treatment with 95% TFA/2.5% trisopropylsilane/2.5% H2O for 2 h at room temperature. Following the filtration of cleavage solution, ice-cold diethyl ether was added to precipitate peptides. Crude peptides were centrifuged 3 × 5 min at 5000 g, washed with diethyl ether, dissolved in 0.1% TFA/50% acetonitrile (ACN)/H2O, and lyophilized.

**Reverse-phase HPLC**

Preparative and analytical RP-HPLC Shimadzu LC-20AT systems equipped with an SPD-20A Prominence UV/VIS detector and a SIL-20AHT autoinjector were used for purification and analysis. An Eclipse XDB-C18 column (Agilent; 7 μm, 21.2 cm × 250 mm, 80 Å, flow rate 8 ml/min) was used for peptide purification. A Zorbax 300SB-C18 column (Agilent; 5 μm, 2.1 × 150 mm, 300 Å, flow rate 1 ml/min) was used to monitor oxidation and analyze the peptide purity. All samples were run from 0 to 60% B in 30 min (solvent A: 0.05% TFA and solvent B: 90% ACN/0.05% TFA). Absorbance was recorded at 214 nm and 280 nm.

**Peptide oxidative folding**

Linear KIIIA was thermodynamically folded as described previously (54). Briefly, linear unprotected KIIIA was dissolved in 0.1 M NH4HCO3, pH 8, at a concentration of 0.3 mg/ml with 100 eq GSH/10 eq GSSG and allowed to stir at room temperature for 24 h.

General procedures for removing cysteine-protecting groups and disulfide bond formation have been previously described by Dekan et al. (Figs. 1A and S1–S3). (63) Briefly, diithiol-containing peptide was dissolved in AcOH (2 mg/ml) following by the dropwise addition of 1 eq of aqueous iodine (I2) in MeOH and kept stirring for 15 min to form the first disulfide bond. Subsequently, water and HCl were added to the above solution so that the final volume was 50% AcOH/50% H2O/0.1% HCl. 8 eq of I2 (dissolved in MeOH) was added to the peptide solution and stirred for 30 min to remove Acm group and form the second disulfide bond. The reactions were monitored by RP-HPLC and mass spectrometry. To stop the reaction, aqueous ascorbic acid was added to quench the I2 until the solution became colorless. The product was isolated by RP-HPLC and lyophilized. The lyophilized bis(Cys(Msbh))-containing peptide was dissolved in TFA (1 mg/ml) and cooled to 0 °C (in an ice bath). Dimethyl sulfide (1% v/v) was added to the stirred solution, followed by NaI (5 eq/sulfoxide group). The solution gradually became yellow. After 15 min, the solution was poured into an ice-cold solution of 10 mM ascorbic acid in H2O (15 × volume of TFA) and the product was isolated by RP-HPLC.

**Liquid chromatography/mass spectrometry**

A Q-Star Pulsar mass spectrometer (SCIEX) equipped with an auto-injector (Agilent Technologies Inc) was used for high-resolution mass analysis. A Zorbax 300SB – C18 column (Agilent; 3.5 μm, 2.1 × 100 mm, 300 Å, flow rate 0.3 ml/min) was used to run all samples. All runs were conducted in solvent A (0.1% formic acid in H2O) and solvent B (aqueous ACN/0.1% formic acid).

**Cell culture and automated patch-clamp electrophysiology**

Automated whole-cell patch-clamp electrophysiology (QPatch-16X; Sophion A/S) was used to examine the activity of KIIIA isoforms at hNaV1.2, hNaV1.4, and hNaV1.7 as previously described (53). hNaV subtypes 1.2, 1.4, and 1.7 (α and β1-subunits) were stably overexpressed in HEK293 cells (SB Drug Discovery). The cells were maintained in Minimum Essential Medium Eagle supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) GlutaMAX, 0.004 mg/ml basicinid, and 0.6 mg/ml genetin.

The intracellular solution contained (in mM) 140 CsF, 1 EGTA, 5 CsOH, 10 Hepes, 10 NaCl, pH 7.3 and adjusted to 320 mOsm with sucrose. The extracellular solution comprised (in mM) 2 CaCl2, 1 MgCl2, 10 Hepes, 4 KCl, 145 NaCl, pH 7.4 and adjusted to 305 mOsm with sucrose. Voltage-clamp experiments were performed in a single-hole configuration mode. KIIIA isoform were diluted in extracellular solution with 0.1% bovine serum albumin. Na+ currents were sampled at 25 kHz and filtered at 8 kHz. The same cells were exposed sequentially to multiples concentrations. Each peptide concentration was incubated for 5 min and the peak current was compared to buffer control. Concentration-response curves were acquired using a holding potential of –90 mV and a 50 ms pulse to –20 mV every 20 s (0.05 Hz). Peak current

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postpeptide addition (I) was normalized to buffer control (I₀). IC₅₀ of IC₅₀ were determined by plotting difference in peak current (I/I₀) and log peptide concentration. Calculated IC₅₀ were compared across subtypes and statistical differences determined by ordinary one-way ANOVA. Concentration-response curves were fitted using the log (inhibitor) versus response-variable slope (four parameters) and analyzed in Prism 8 (GraphPad Software).

NMR and 3D structure calculation

Peptides were dissolved in 500 µl MilliQ water (MilliPore) and 50 µl D₂O (Cambridge isotopes). A Bruker 600 MHz Avance III spectrometer equipped with a cryoprobe (Bruker) was used to acquire NMR spectra, as described by Agwa et al. (83) 1D ¹H, and 2D ¹H-¹H TOCSY (80 ms mixing time) and ¹H-¹H NOESY (200 ms mixing time), natural abundance ¹H-¹5N HSQC and D₂O exchange 2D ¹H-¹H TOCSY and ¹H-¹³C HSQC were collected (84, 85). Spectra were processed using TopSpin 3.5 (Bruker) and CCPNMR Analysis 2.4.1 (CCPN, University of Cambridge) (45, 46). The chemical shift of water at 4.76 ppm was used as a reference (86).

Dihedral angles were identified using TALOS-N (87), and initial 3D structures were calculated using the AUTO and ANNEAL functions in CYANA (88) followed by refinement in a watershell using CNS (47, 89). Additional H-bond restraints were included derived from temperature coefficient experiments in combination with D₂O exchange data (90). Fifty structures were calculated and the best 20 structures (based on energy and MolProbity scores (49)) were kept as the final 3D structure. Molmol (48) was used for visualization and RMSD calculations.

Docking and protein–protein interaction analysis

Autodock VINA software (51) assisted by MGLTools (91) was used for molecular docking of KIIIA isomers in human NaV1.2 β2 cryo-EM structure (PDB 68E) (50). To define the search space of the hNaV1.2 structure, a grid box with the following dimensions: center x = 143.432, center y = 136.254, and center z = 155.036 was used. The size of the grid box for all the docking in hNaV1.2 was as follows: size x = 30, size y = 30, and size z = 30. The exhaustiveness for the search was set to 8. The lowest energy models were submitted and analyzed by PBDSum (52) for protein–protein interactions. PyMol was used for visualization.

Data availability

Supporting information includes orthogonal peptide oxidation, statistical analysis of NMR solution structures of Isomer 2 and Native KIIIA, regioselective oxidation, 1D and 2D ¹H NMR on Isomer 1, Isomer 2, and Native KIIIA, and putative-binding interactions between NaV₁.2 and Isomer 1, Isomer 2 and Native KIIIA can be found online. NMR coordinates for Isomer 2, and Native KIIIA solution structures have been submitted to the Protein Data Bank (KIIIA Isomer 2 PDB ID: 7SAW and KIIIA Native PDB ID: 7SAV) and the BioMagnetic Resonance Bank (KIIIA Isomer 2 BMRB: 30954 and KIIIA Native BMRB: 30953). All other data are included in the main article.

Supporting information—This article contains supporting information (34, 52, 63, 68–70, 86).

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Abbreviations—The abbreviations used are: Acm, acetamidomethyl; ACN, acetonitrile; Fmoc, 9-fluorenylmethoxycarbonyl; Mebzl, 4-methylbenzyl; Mob, 4-methoxybenzyl; Msbb, 4,4’-dimethylsulfonylbenzhydryl; Na₅, voltage-gated sodium channel; PDB, Protein data bank; RP-HPLC, reverse-phase HPLC; Trt, S-triphenylmethyl.

References

1. Dib-Hajj, S. D., Black, J. A., and Waxman, S. G. (2009) Voltage-gated sodium channels: Therapeutic targets for pain. Pain Med. 10, 1260–1269
2. Minett, M. S., Pereira, V., Skandar, S., Matsuyama, A., Lollignier, S., Kanellopoulos, A. H., Mancini, F., Iannetti, G. D., Bogdanow, Y. D., Santana-Varela, S., Millet, Q., Baskozos, G., MacAllister, R., Cox, J. J., Zhao, J., et al. (2015) Endogenous opioids contribute to insensitivity to pain in humans and mice lacking sodium channel Na₅₁.7. Nat. Commun. 6, 1–16
3. Dib-Hajj, S. D., Black, J. A., and Waxman, S. G. (2015) Na₅₁.9: A sodium channel linked to human pain. Nat. Rev. Neurosci. 16, 511–519
4. Osteen, J. D., Herzig, V., Gilchrist, J., Emrick, J. J., Zhang, C., Wang, X., Castro, J., Garcia-Caraballo, S., Grundy, L., Rychkov, G. Y., Weyer, A. D., Dekan, Z., Undheim, E. A., Aledor, P., Stucky, C. L., et al. (2016) Selective spider toxins reveal a role for the Na₅₁.1 channel in mechanical pain. Nature 534, 494–499
5. Vetter, I., Deuis, J. R., Mueller, A., Israel, M. R., Starobova, H., Zhang, A., Rash, L. D., and Mobi, M. (2017) Na₅₁.7 as a pain target - from gene to pharmacology. Pharmacol. Ther. 172, 73–100
Inhibition of NaV channels by KIIIA disulfide isomers

6. Bayurt, T. H., and Sligar, S. G. (2002) Single-molecule height measurements on microsomal cytochrome P450 in nanometer-scale phospholipid bilayer disks. *Epilepsy Res.* 99, 6725–6730
7. Remme, C. A., and Bezzina, C. R. (2010) Sodium channel (dy)sfunction and cardiac arrhythmias. *Cardiovasc. Ther.* 28, 287–294
8. Catterall, W. A., Goldin, A. L., and Waxman, S. G. (2005) International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol. Rev.* 57, 397–409
9. Weiss, L. A., Escayg, A., Kearney, J. A., Trudeau, M., MacDonald, B. T., Mori, M., Reichert, J., Buxbaum, J. D., and Meisler, M. H. (2003) Sodium channels SCN1A, SCN2A and SCN3A in familial autism. *Mol. Psychiatry* 8, 186–194
10. Planells-Cases, R., Caprini, M., Zhang, J., Rockenstein, E. M., Rivera, R. R., Boucher, T. J., Okuse, K., Bennett, D. L., Munson, J. B., Wood, J. N., and Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W. A. (2011) The Zhang, M. M., Han, T. S., Olivera, B. M., Bulaj, G., and Yoshikami, D. (1998) μ-Conotoxin PIIIA, a new peptide for discriminating among tetrodotoxin-sensitive Na channel subtypes. *J. Neurosci.* 18, 4473–4481
11. Tietze, A. A., Tietze, D., Ohlenschlager, O., Leipold, E., Ullrich, F., Kuhl, T., Mischo, A., Buntkowsky, G., Gorlach, M., Heinemann, S. H., and Imhof, D. (2012) Structurally diverse μ-conotoxin PIIIA isomers block sodium channel Nav,1.4 *Angew. Chem. Int. Ed. Engl.* 51, 4058–4061
12. Dutton, J. L., Bansal, P. S., Hogg, R. C., Adams, D. J., Aulewo, P. F., and Craik, D. J. (2002) A new level of conotoxin diversity, a non-native disulfide bond connectivity in α-conotoxin AuIB reduces structural definition but increases biological activity. *J. Biol. Chem.* 277, 48489–48857
13. Wilson, M. J., Yoshikami, D., Azam, L., Gajewiak, J., Olivera, B. M., Bulaj, G., and Zhang, M. M. (2011) μ-Conotoxins that differentially block sodium channels Nav,1.1 through 1.8 identify those responsible for action potentials in sciatic nerve. *Proc. Natl. Acad. Sci. U. S. A.* 108, 10302–10307
14. Knapp, O., McArthur, J. R., and Adams, D. J. (2012) Conotoxins targeting neuronal voltage-gated sodium channel subtypes: Potential analgesics? *Toxins* 4, 1236–1260
15. Bulaj, G., West, P. J., Garrett, J. E., Watkins, M., Zhang, M. M., Norton, R. S., Smith, B. J., Yoshikami, D., and Olivera, B. M. (2005) Novel conotoxins from Conus striatus and Conus kinositai selectively block TTX-resistant sodium channels. *Biochemistry* 44, 7259–7265
16. Stevens, M., Peigneur, S., and Tytgat, J. (2011) Neurotoxins and their binding areas on voltage-gated sodium channels. *Front. Pharmacol.* 2, 71
17. Akopian, A. N., Souslova, V., England, S., Okuse, K., Mcmahon, S. B., Boyce, S., Dickenson, A. H., and Nal, W. (1999) The TTX-R sodium channel SNS has a specialized function in pain pathways. *Nat. Neurosci.* 2, 461–465
18. Lehmann-Horn, F., Jurkat-Rott, K., and Rudel, R. (2002) Periodic paralys: Understanding channelopathie. *Curr. Neural. Neurosci. Rep.* 2, 61–69
19. Takahata, T., Yasui-Furukori, N., Sasaki, S., Igarashi, T., Okunuma, K., Mukakata, A., and Tateishi, T. (2003) Nucleotide changes in the translated region of SCNSNA from Japanese patients with Brugada syndrome and control subjects. *Life Sci.* 72, 2391–2399
20. Yang, Y., Wang, Y., Li, S., Xu, Z., Li, H., Ma, L., Fan, J., Bu, D., Liu, B., Fan, Z., Wu, G., Jin, J., Ding, B., Zhu, X., and Shen, Y. (2004) Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary erythermalgia. *J. Med. Genet.* 41, 171–174
21. Akopian, A. N., Souslova, V., England, S., Okuse, K., Mcmahon, S. B., Boyce, S., Dickenson, A. H., and Nal, W. (1999) The TTX-R sodium channel SNS has a specialized function in pain pathways. *Nat. Neurosci.* 2, 541–548
22. Hoffmann, T., Sharon, O., Wittmann, J., Carr, R. W., Vlyshnevskaya, A., Col, R., Nassar, M. A., Reeh, P. W., and Weidner, C. (2018) Na1.7 and pain: Contribution of peripheral nerves. *Pain* 159, 496–506
23. Maxwell, M., Undheim, E. A. B., and Mobili, M. (2018) Secreted cysteine-rich repeat proteins "SCREPs": A novel multi-domain architecture. *Front. Pharmacol.* 9, 1–16
24. Zhang, M. M., Han, T. S., Olivera, B. M., Bulaj, G., and Yoshikami, D. (2010) μ-Conotoxin KIIIA derivatives with divergent affinities versus efficacies in blocking voltage-gated sodium channels. *Biochemistry* 49, 4804–4812
25. Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W. A. (2011) The crystal structure of a voltage-gated sodium channel. *Nature* 475, 533–538
26. Terlau, H., and Olivera, B. M. (2004) Conus venoms: A rich source of novel ion channel-targeted peptides. *Physiol. Rev.* 84, 41–68
27. Kaas, Q., Westermann, J. C., Halai, R., Wang, C. K., and Craik, D. J. (2008) ConoServer, a database for conopeptide sequences and structures. *Bioinformatics* 24, 445–456
28. Kaas, Q., Westermann, J. C., and Craik, D. J. (2010) Conopeptide characterization and classifications: An analysis using ConoServer. *Toxicon* 55, 1491–1509
29. Corpuz, G. P., Jacobsen, R. B., Jimenez, E. C., Watkins, M., Walker, C., Colledge, C., Garrett, J. E., McDougal, O., Li, W., Gray, W. R., Hillyard, D. R., Rivier, J., McIntosh, J. M., Cruz, L. J., and Olivera, B. M. (2005) Definition of the M-conotoxin superfamily: Characterization of novel peptides from molluscicorous Conus venoms. *Biochemistry* 44, 8176–8186
30. Shon, K.-J., Olivera, B. M., Watkins, M., Jacobsen, R. B., Gray, W. R., Floresca, C. Z., Cruz, L. J., Hillyard, D. R., Brink, A., Terlau, H., and Yoshikami, D. (1998) μ-Conotoxin PIIIA, a new peptide for discriminating among tetrodotoxin-sensitive Na channel subtypes. *J. Neurosci.* 18, 4473–4481
31. Miljanich, G. P. (2004) Ziconotide: Neuronal calcium channel blocker for treatment of chronic pain. *Future Med. Chem.* 6, 1677–1698
32. Barton, M. E., and White, H. S. (2004) The effect of CGX-1007 and CI-1041, novel NMDA receptor antagonists, on kindling acquisition and expression. *Epilepsy Res.* 59, 1–12
33. Cruz, L. J., Kupryszewski, G., Lecheminant, G. W., Gray, W. R., Olivera, B. M., and Rivier, J. (1989) μ-Conotoxin GiIA, a peptide ligand for muscle sodium channels: Chemical synthesis, radiolabeling, and receptor characterization. *Biochemistry* 28, 3437–3442
34. Green, B. R., Bulaj, G., and Norton, B. S. (2014) Structure and function of μ-conotoxins, peptide-based sodium channel blockers with analgesic activity. *Future Med. Chem.* 6, 1029–1040
35. Sharpe, I. A., Palant, E., Schroeder, C. I., Kaye, D. M., Adams, D. J., Aulewo, P. F., and Lewis, R. J. (2003) Inhibition of the norepinephrine transporter by the venom peptide χ-MRF. *Science* 298, 40317–40323
36. Staats, P. S., Yearwood, T., Charapata, S. G., Presley, R. W., Wallace, M. S., Byas-Smith, M., Fisher, R., Bryce, D. A., Mangieri, E. A., Luther, R. R., Mayo, M., McGuire, D., and Ellis, D. (2004) Intrathecal ziconotide in the
Inhibition of NaV channels by KIIIA disulphide isomers

treatment of refractory pain in patients with cancer or AIDS: A randomized controlled trial. JAMA 291, 63–70

45. Wu, K. (1986) NMR of Proteins and Nucleic Acids, Wiley Interscience, New York, NY

46. Vranken, W. F., Boucher, W., Stevens, T. J., Fogh, R. H., Pajon, A., Llinas, M., Ulrich, E. L., Markley, J. L., Ionides, I., and Laue, E. D. (2005) The CCPN data model for NMR spectroscopy: Development of a software pipeline. Proteins 59, 687–696

47. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. 54, 905–921

48. Koradi, R., Billeter, M., and Wuthrich, K. (1996) MOLMOL: A program for display and analysis of macromolecular structures. J. Mol. Graph. 14, 29–32

49. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: All-atom structure validation for macromolecular crystallography. Acta Crystallogr. D Biol. Crystallogr. 66, 12–21

50. Pan, X., Li, Z., Huang, X., Huang, G., Gao, S., Shen, H., Liu, L., Lei, J., and Yan, N. (2019) Molecular basis for pore blockade of human Na(+) channel NaV1.2 by the μ-conotoxin KIIIA. Science 363, 1309–1313

51. Trotz, O., and Olson, A. J. (2010) AutoDock vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 31, 455–461

52. Laskowski, R. A., Jarlskog, J., Pravda, V., Varekova, R. S., and Thornton, J. M. (2018) PDBsum: Structural summaries of PDB entries. Protein Sci. 27, 129–134

53. McMahon, K. L., Tran, H. N. T., Deuis, J. R., Lewis, R. J., Vetter, I., and Schroeder, C. I. (2020) Discovery, pharmacological characterisation and NMR structure of the novel μ-conotoxin SaDCT, a potent and irreversible Na(+) channel inhibitor. BioMedicines 8, 391–406

54. Tran, H. N. T., Tran, P., Deuis, J. R., Agwa, A. J., Zhang, A. H., Vetter, I., and Schroeder, C. I. (2020) Enzymatic ligation of a pore blocker toxin and a gating modifier toxin: Creating double-knotted peptides with improved sodium channel NaV1.7 inhibition. Bioconjug. Chem. 31, 64–73

55. Knuhtsen, A., Whiting, R., McMwhiney, F. S., Whitmore, C., Smith, B. O., Green, A. C., Timperley, C. M., Kinnear, K. L., and Jamieson, A. G. (2020) μ-Conotoxin KIIIA peptidomimetics that block human voltage-gated sodium channels. Pept. Sci. 113, 1–11

56. Eliaen, R., Andresen, T. L., and Conde-Frieboes, K. W. (2012) Handling a tricyclic: Orthogonal versus random oxidation of the tricyclic inhibitor cystine knotted peptide gujarmin. Peptides 37, 144–149

57. Wu, F., Mayer, J. P., Gelfanov, V. M., Liu, F., and DiMarchi, R. D. (2017) Deuis, J., Motlib, M., Pennington, M. W., Fung, E., Nemeth, E., and Alewood, P. F. (2014) Total synthesis of human hepcidin through regioselective disulphide-bond formation by using the safety-catch cysteine protecting group 4,4'-dimethylsulfonylbenzhydrazyl. Angew. Chem. Int. Ed. Engl. 53, 2931–2944

58. Kluver, E., Schulz-Marone, S., Scheid, S., Meyer, B., Forssmann, W. G., and Adermann, K. (2005) Structure-activity relation of human β-defensin 3: Influence of disulphide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. Biochemistry 44, 9804–9816

59. Schulz, A., Kluver, E., Schulz-Marone, S., and Adermann, K. (2005) Engineering disulphide bonds of the novel human β-defensins hBD-2 and hBD-3: Differences in disulphide formation and biological activity among human β-defensins. Biopolymers 80, 34–49

60. Szabo, I., Schlosser, G., Hudec, F., and Mezo, G. (2007) Disulphide bond rearrangement during regioselective oxidation in PhS(O)Ph/CH2SiCl3 mixture for the synthesis of α-conotoxin GI. Biopolymers 88, 20–28

61. Denis, B. and Trifilieff, E. (2000) Synthesis of palmityl-thioester T-cell epitopes of myelin proteolipid protein (PLP). Comparison of two thiol protecting groups (StBu and Mmt) for on-resin acylation. J. Pept. Sci. 6, 372–377

62. Norton, R. S., and Pallaghy, P. K. (1998) The cystine knot structure of ion channel toxins and related polypeptides. Toxicon 36, 1573–1583

63. Pallaghy, P. K., Nielsen, K. I., Craik, D. J., and Norton, R. S. (1994) A common structural motif incorporating a cystine knot and a triple-stranded beta-sheet in toxic and inhibitory polypeptides. Protein Sci. 3, 1833–1839

64. Agwa, A. J., Tran, P., Mueller, A., Tran, H. N. T., Deuis, J. R., Israel, M. R., McMahon, K. L., Craik, D. J., Vetter, I., and Schroeder, C. I. (2020) Manipulation of a spider peptide toxin alters its affinity for lipid bilayers and potency and selectivity for voltage-gated sodium channel subtype 1.7. J. Biol. Chem. 295, 5067–5080

65. Markgraf, R., Leipoldt, F., Schirmeyer, J., Paolini-Bertrand, M., Hartley, O., and Heinemann, S. H. (2012) Mechanism and molecular basis for the sodium channel subtype specificity of μ-conopeptide CnIIIIC. Br. J. Pharmacol. 167, 576–586

66. Van Der Haegen, A., Peigneur, S., and Tytgat, I. (2011) Importance of position 8 in μ-conotoxin KIIIA for voltage-gated sodium channel selectivity. FEBS J. 278, 3408–3418

67. Catterall, W. A. (2000) From ionic currents to molecular mechanisms: The structure and function of voltage-gated sodium channels. Neuron 26, 13–25

68. He, B., and Soderlund, D. M. (2014) Functional expression of rat Na+,1.6 voltage-gated sodium channels in HEK293 cells: Modulation by the auxiliary β1 subunit. PLoS One 9, e85188

69. Goldin, A. L., Barchi, R. L., Caldwell, J. H., Hofmann, F., Howe, J. R., Hunter, J. C., Kallen, R. G., Mandel, G., Messler, M. H., Netter, Y. B., Noda, M., Tamkun, M. M., Waxman, S. G., Wood, J. N., and Catterall, W. A. (2000) Nomenclature of voltage-gated sodium channels. Neuron 28, 365–369

70. West, P. J., Bulaj, G., Garrett, J. E., Olivera, B. M., and Yoshikami, D. (2002) μ-conotoxin SnIIIIC, a potent inhibitor of tetrodotoxin-resistant sodium channels in amphibian sympathetic and sensory neurons. Biochemistry 41, 15388–15393

71. Keizer, D. W., West, P. J., Lee, E. F., Yoshikami, D., Olivera, B. M., Bulaj, G., and Norton, R. S. (2003) Structural basis for tetrodotoxin-resistant sodium channel binding by μ-conotoxin SnIIIIC. J. Biol. Chem. 278, 46805–46813

72. Gilchrist, J., Das, S., Van Petegem, F., and Bosmans, F. (2013) Crystallographic insights into sodium-channel modulation by the β4 subunit. Proc. Natl. Acad. Sci. U. S. A. 110, 5016–5024

73. Namadurai, S., Yereddi, N. R., Cusdin, F. S., Huang, C. L., Chirgadze, D. Y., and Jackson, A. P. (2015) A new look at sodium channel β subunits. Biochim. Biophys. Acta 1850, 1–12

74. Zhang, M. M., Wilson, M. J., Azam, L., Gajewiak, J., Rivier, J. E., Bulaj, G., Olivera, B. M., and Yoshikami, D. (2013) Co-expression of NaV β subunits
alters the kinetics of inhibition of voltage-gated sodium channels by pore-blocking \(\mu\)-conotoxins. Br. J. Pharmacol. 168, 1597–1610

81. Gajewiak, J., Azam, L., Imperial, J., Walewska, A., Green, B. R., Bandypadhyay, P. K., Raghuraman, S., Ueberheide, B., Bern, M., Zhou, H. M., Minassian, N. A., Hagan, R. H., Flinspach, M., Liu, Y., Bulaj, G., et al. (2014) A disulfide tether stabilizes the block of sodium channels by the conotoxin \(\mu\)0 section sign-GVIIJ. Proc. Natl. Acad. Sci. U. S. A. 111, 2758–2763

82. Zhang, M. M., Green, B. R., Catlin, P., Fiedler, B., Azam, L., Chadwick, A., Terlau, H., McArthur, J. R., French, R. J., Gulys, J., Rivier, J. E., Smith, B. J., Norton, R. S., Oliveira, B. M., Yoshikami, D., et al. (2007) Structure/function characterization of \(\mu\)-conotoxin KIIIA, an analgesic, nearly irreversible blocker of mammalian neuronal sodium channels. J. Biol. Chem. 282, 30699–30706

83. Agwa, A. J., Blomster, L. V., Craik, D. J., King, G. F., and Schroeder, C. I. (2018) Efficient enzymatic ligation of inhibitor cystine knot spider venom peptides: Using sortase a to form double-knottins that probe voltage-gated sodium channel Nav1.7. Bioconjug. Chem. 29, 3309–3319

84. Braunschweiler, L., and Ernst, R. R. (1983) Coherence transfer by isotropic mixing - application to proton correlation spectroscopy. J. Magn. Reson. 53, 521–528

85. Jeener, J., Meier, B. H., Bachmann, P., and Ernst, R. R. (1979) Investigation of exchange processes by two-dimensional NMR spectroscopy. J. Chem. Phys. 71, 4546–4553

86. Gottlieb, H. E., Kotlyar, V., and Nudelman, A. (1997) NMR chemical shifts of common laboratory solvents as trace impurities. J. Org. Chem. 62, 7512–7515

87. Shen, Y., and Bax, A. (2013) Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. J. Biomol. NMR 56, 227–241

88. Gottstein, D., Kirchner, D. K., and Guntert, P. (2012) Simultaneous single-structure and bundle representation of protein NMR structures in torsion angle space. J. Biomol. NMR 52, 351–364

89. Brunger, A. T. (2007) Version 1.2 of the crystallography and NMR system. Nat. Prot. 2, 2728–2733

90. Baxter, N. J., and Williamson, M. P. (1997) Temperature dependence of \(^1\)H chemical shifts in proteins. J. Biomol. NMR 9, 359–369

91. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. 30, 2785–2791

92. Wang, C. Z., Zhang, H., Jiang, H., Lu, W., Zhao, Z. Q., and Chi, C. W. (2006) A novel conotoxin from Conus striatus, \(\mu\)-SIIIA, selectively blocking rat tetrodotoxin-resistant sodium channels. Toxicon 47, 122–132

93. Schroeder, C. I., Ekberg, J., Nielsen, K. J., Adams, D., Loughnahan, M. L., Thomas, L., Adams, D. J., Llewellyn, P. F., and Lewis, R. J. (2008) Neuronally \(\mu\)-conotoxins from Conus striatus utilize an \(\alpha\)-helical motif to target mammalian sodium channels. J. Biol. Chem. 283, 21621–21628

94. Cruz, L. J., Gray, W. R., Oliveira, B. M., Zekus, R. D., Kerr, L., Yoshikami, D., and Moczydlowski, E. (1985) Conus geographus toxins that discriminate between neuronal and muscle sodium channels. J. Biol. Chem. 260, 9280–9288

95. Holford, M., Zhang, M. M., Gowd, K. H., Azam, L., Green, B. R., Watkins, M., Owby, J. P., Yoshikami, D., Bulaj, G., and Olivera, B. M. (2009) Pruning nature: Biodiversity-derived discovery of novel sodium channel blocking conotoxins from Conus bullatus. Toxicon 53, 90–98

96. Zhang, M. M., Fiedler, B., Green, B. R., Catlin, P., Watkins, M., Garrett, J. E., Smith, B. J., Yoshikami, D., Olivera, B. M., and Bulaj, G. (2006) Structural and functional diversities among \(\mu\)-conotoxins targeting TTX-resistant sodium channels. Biochemistry 45, 3723–3732