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Expanding Chemical Diversity of Conotoxins: Peptoid-Peptide Chimeras of the Sodium Channel Blocker μ -KIIIA and its Selenopeptide Analogues

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Abstract

The μ -conotoxin KIIIA is a three disulfide-bridged blocker of voltage-gated sodium channels (VGSCs). The Lys⁷ residue in KIIIA is an attractive target for manipulating the selectivity and efficacy of this peptide. Here, we report the design and chemical synthesis of μ -cono-peptoid analogues (peptomers) in which we replaced Lys⁷ with peptoid monomers of increasing side-chain size: *N*-methylglycine, *N*-butylglycine and *N*-octylglycine. In the first series of analogues, the peptide core contained all three disulfide bridges; whereas in the second series, a disulfide-depleted selenoconopeptide core was used to simplify oxidative folding. The analogues were tested for functional activity in blocking the Na_v1.2 subtype of mammalian VGSCs exogenously expressed in *Xenopus* oocytes. All six analogues were active, with the *N*-methylglycine analogue, [Sar⁷]KIIIA, the most potent in blocking the channels while favoring lower efficacy. Our findings demonstrate that the use of *N*-substituted Gly residues in conotoxins show promise as a tool to optimize their pharmacological properties as potential analgesic drug leads.

Keywords

μ -conotoxins; KIIIA, peptomers; selenocysteine; diselenide; sodium channels; electrophysiology

1. Introduction

μ -Conotoxin KIIIA is a 16-amino acid analgesic peptide that blocks voltage-gated sodium channels (Figure 1). Structure-activity relationship (SAR) studies identified several residues

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as hot spots in determining the selectivity of KIIIA [1, 2, 3, 4, 5]. Initial work pointed towards Lys⁷ as a critical residue for blocking the neuronal subtype Na_v1.2 and the skeletal muscle subtype Na_v1.4 [1, 3, 4], while more recent studies also suggested a contribution of Trp⁸ [5]. A study that investigated molecular interactions between KIIIA and the sodium channels implicated four residues, Lys⁷Arg¹⁰His¹² and Lys¹⁴ that could interact with the pore-forming P-loops I and III [4]. It is known that the positively charged residues of the μ -conotoxin interact with negatively charged residues that form so-called inner (DEKA) and outer (EEDD) ring [6, 7]. While the studies show that there is more than one binding mode in which positively charged residues of the conotoxin contribute to blocking sodium currents, the critical role of the conserved charged residue in the second intercysteine loop in KIIIA (Lys⁷), GIIIA (Arg¹³) and PIIIA (Arg¹⁴), is well documented [1, 2, 3, 4, 8].

The studies with KIIIA analogues examined structural factors responsible for affinity (K_d or IC_{50}) versus efficacy (the fraction of sodium current blocked at saturating ligand concentrations) in inhibiting sodium channels [3]. Both of these parameters are expected to play an important role in developing safe analgesics. The affinity as well as efficacy of native KIIIA was high toward Na_v1.2 with $K_d = 0.0054 \mu M$ and efficacy $\sim 95\%$ (i.e., residual current, $rI_{Na} \sim 5\%$), respectively. Analogues of disulfide-depleted KIIIA in which Lys⁷ was replaced with small amino acids such as Gly, Ala or Ser, had lower efficacies than that of native KIIIA toward Na_v1.2. However, the affinities of these analogues were poor ($K_d = 0.14\text{--}0.56 \mu M$). In contrast, analogues with amino acids having larger side-chains (Leu, Phe) in position 7 showed higher efficacy but lower affinity toward sodium channel Na_v1.2 [3]. It is important to mention here that ideal KIIIA-derived drug leads for the treatment of pain should exhibit: (1) relatively low efficacy that will provide higher safety margin for blocking sodium currents, and (2) high affinities for relevant neuronal sodium channel subtypes involved in nociception [9], while lacking apparent affinities for the skeletal muscle and cardiac subtypes.

To further examine the critical role of Lys⁷ residue in blocking Na_v1.2, we designed peptoid analogues of KIIIA where the *N*-substituted glycine residue varied in side-chain size. Our rationale was that replacing Lys⁷ with peptoid monomers may lead to a lower efficacy in blocking sodium currents, while offering some of below-mentioned advantages of peptoids. Peptoids are composed of *N*-substituted glycine units (peptoid monomers) and mimic α -peptides in which the side chains are attached to the backbone amide nitrogens instead of the α -carbons [10]. Peptoids can play an important role as prospective therapeutics in view of their improved cellular permeabilities [11, 12] and superior serum stability [13]. Recently, the *N*-substituted glycine residues were used to optimize the subtype selectivity of neurotensin analogues [14], expanding the applications of peptide-peptoid chimeras (*peptomers* as they were referred to by Ostergaard and Holm [15]). Peptoids can be generated by a straightforward submonomer-synthesis method [16], which allows the incorporation of a variety of amines that are commercially available [17].

To accelerate SAR studies of disulfide-rich peptides, recent efforts have focused on applying the selenopeptide technology to simplify oxidative folding [18, 19, 20, 21, 22, 23, 24, 25]. This technology was applied towards disulfide-depleted analogues, as was shown for μ -conotoxin KIIIA [26] and ω -conotoxin GVIA [27]. In this strategy, one disulfide bridge is

eliminated by replacing a pair of Cys residues with Ala residues, the second disulfide bridge is replaced with a diselenide bridge, while the remaining third bridge is retained; thus, the number of disulfide bridges is reduced from three to one. The presence of only one disulfide and one diselenide bridge decreases the number of potential oxidized peptide isomers from potentially 15 to a single product that is favored by a redox preference of diselenide over disulfide bond, thereby simplifying the oxidative folding. In view of the advantage offered by selenopeptides, we applied this strategy to generate peptoid analogues that had a disulfide-deficient diselenide KIIIA core. In this work, we synthesized and characterized peptomer analogues of KIIIA and its disulfide-deficient form in which Lys⁷ was replaced with one of three *N*-substituted glycine residues: *N*-methylglycine (Sar), *N*-butylglycine (Nnle) and *N*-octylglycine (Noct) (Figure 1).

2. Results and Discussion

Here, we report the design, synthesis and bioactivity of six analogues of μ -conopeptoid KIIIA in which we replaced Lys⁷ with peptoid monomers of increasing side-chain size: *N*-methylglycine (Sar), *N*-butylglycine (Nnle) and *N*-octylglycine (Noct) (Figure 1A). The core of the first set of three μ -conopeptoid analogues contained three disulfide bridges, while the second set of three conopeptoid analogues had as their core a disulfide-deficient diselenide form of KIIIA. The core structures of these analogues are shown in Figure 1B.

Chemical synthesis and oxidative folding of peptide-peptoid chimeras of KIIIA are shown in Figure 2 and 3. The μ -conopeptoid analogues were synthesized using the Fmoc protocols as described previously [16, 26]. The KIIIA analogues with Nnle and Noct residues in position 7 were incorporated using submonomer approach [16], as fully described in the Experimental Section and illustrated in Figure 2A. In both series of conopeptoid analogues, the cysteine thiols were protected with trityl (Trt) groups, whereas in the second series of disulfide-deficient selenoconopeptoid analogues, the selenocysteine selenols were protected with *p*-methoxybenzyl (Mob) groups (Figure 2B and 2C) [28, 29, 43]. The protected groups were removed during the cleavage of the peptides from the resin with a reagent K or (for selenocysteine containing analogues) an enriched reagent K with 1.3 eq DTNP. The presence of DTNP in the cleavage mixture is critical for the removal of the *p*-methoxybenzyl groups and a simultaneous formation of a diselenide bridge.

To carry out oxidative folding, all synthesized μ -conopeptoid analogues were treated with 0.1 M Tris-HCl, 1mM EDTA (pH 7.5) and a mixture of the 1 mM oxidized glutathione (GSSG) and 1 mM reduced glutathione (GSH) for 60 min, followed by quenching with acetic acid and separations by HPLC. It was shown previously that such oxidizing conditions were optimal for formation of the native disulfide bridges in μ -conotoxins [30]. As illustrated in Figure 3A, for [Sar⁷]KIIIA, [Nnle⁷]KIIIA and [Noct⁷]KIIIA the oxidative folding yielded two major products. Both major peaks of each analogue were separated on HPLC C₈ column and analyzed by MALDI-TOF mass spectrometry. Both oxidation products of each three disulfide bridged peptomer KIIIA analogue had the same mass, corresponding to fully oxidized form. For each analogue, the folding species eluted as a first peak (marked with asterisk in Figure 3A) retained the activity in blocking Na_v1.2. The identities of the folded products were confirmed by MALDI-TOF or ESI mass spectrometry

(Table 1). The folding yields for [Sar⁷]KIIIA, [Nnle⁷]KIIIA and [Noct⁷]KIIIA, calculated from integration of HPLC peaks, obtained 20%, 21% and 21%, respectively.

To produce KIIIA peptoid analogues with simplified folding properties, we designed three disulfide-depleted selenopeptoid KIIIA analogues (Figure 1B). Our study showed no significant differences between the three-disulfide bridged [Ala⁷]KIIIA and the disulfide-depleted [Ala^{1,7,9}]KIIIA in potencies and residual currents in blocking Na_v1.2 [33], while recently described disulfide-depleted analogues of KIIIA lacking Cys¹ and Cys⁹ exhibited a potent block of Na_v1.2 [31]. Interestingly, while KIIIA was previously reported to share the disulfide pattern typical of the μ -conotoxin family (i.e., 1st Cys to 4th Cys, 2nd to 5th3rd to 6th) [32, 33], the disulfide connectivity of the synthetic peptide was recently shown as: 1st to 5th2nd to 4th3rd to 6th [34, 35]. Given our previous functional results with [Ala^{1,7,9}]KIIIA and [Ala^{1,9}Asp⁷]KIIIA [3], we designed the disulfide-depleted analogues by a removal of the first pair of cysteine residues (Cys¹ and Cys⁹), while the second pair (Cys² and Cys¹⁵) was replaced with selenocysteines because this substitution simplifies the oxidative folding of KIIIA [26] and another μ -conotoxin, SIIIA [36]. The third cysteine pair (Cys⁴ and Cys¹⁶), which was critical for the biological activity [37] was unchanged.

The synthesis of KIIIA analogues comprising Nnle and Noct residues required application of a two-step, submonomeric method [16], whereas Sar was incorporated into the peptide chain as an Fmoc derivative. When the solid-phase synthesis was completed, the crude conopeptoid analogues containing selenocysteine residues were treated with 50 mM DTT in Tris-HCl buffer (pH 7.5) to form the diselenide bridge spontaneously [20, 23, 26]. In the next step, oxidative folding was carried out, and each reaction yielded a single peak (Figure 3B). Our previous studies also showed that the oxidative folding of disulfide-deficient selenoconotoxin KIIIA analogues lead to one major product [26].

All six conopeptoid analogues retained activity in blocking Na_v1.2, each with different kinetics and affinities (Figure 4, Table 2). The three disulfide-bridged peptomer KIIIA analogues had slower on rates than native KIIIA. [Noct⁷]KIIIA was the slowest blocker with an on rate 10-fold slower than that of KIIIA. The off rates of these three analogues were about 3- to 5-fold faster than that of native KIIIA. So they have at least a 10-fold lower affinity than KIIIA. The three disulfide-deficient selenopeptoid KIIIA analogues had similar on rates as that of KIIIA, but they had off rates at least 100-fold faster than that of KIIIA. Therefore, they had at least a 100-fold lower affinity than that of KIIIA. These results are consistent with those previously acquired by our group; i.e., when with the first disulfide bridge was deleted, KIIIA always had faster off rate than that of native peptide [37]. When [Sar⁷]KIIIA was tested at concentrations higher than 10 μ M, the block remained near 70% (not shown), indicating that the [Sar⁷]KIIIA's efficacy was only about 70%; which is consistent with the observation that the other two analogues, [Noct⁷]KIIIA and [Nnle⁷]KIIIA, each at 10 μ M blocked 80%, indicating that [Sar⁷]KIIIA was the least efficacious of the three.

For all six peptoid KIIIA analogues, increases in the residual sodium currents (from ~20% for *N*-butylglycine and *N*-octylglycine to ~30% for *N*-methylglycine) were generally correlated with decreasing side-chain size of peptoid monomers. This observation is in

agreement with our previous results with disulfide-deficient KIIIA peptidic analogues containing Lys⁷ substitutions [3]. In that study, the residual sodium currents were inversely correlated with the size of the side chain of the residue in position 7, but not with its electrical charge or hydrophobicity. Noteworthy, the Na_v1.2[F385C] mutant was shown to have a two-fold increased K_d for KIIIA and a 2-fold increased residual current with KIIIA [2]. Our current results are difficult to interpret in the context of molecular interactions between KIIIA and Na_v1.2, given dynamic interactions between TTX and KIIIA at the pore of the channel [2, 38]. We speculate here that the peptoid monomers in position 7 may also interact with a pore region of Na_v1.2 in a similar way as the molecular interactions identified between native KIIIA and Na_v1.2 [4].

This work confirmed that the substitutions of Lys⁷ with a peptide or peptoid monomer that vary in size offer an opportunity to study the safety and utility of sodium channel blockers for neurological disorders. As discussed previously [3], sodium channel blockers that exhibit high potency and low efficacy (high residual currents) provide attractive drug leads to control neuronal hyperexcitability mediated by sodium channels. Lowering the efficacy in [Sar⁷]KIIIA by 20% is a promising step towards developing μ -conotoxin based leads as analgesics. KIIIA also is a blocker of Na_v1.7 [39], a sodium channel subtype that has become a prime molecular target for the treatment of pain [40]. While R14A mutation in KIIIA increased its selectivity towards Na_v1.7 [4] more SAR studies are needed to minimize the current high potency of KIIIA in blocking Na_v1.4, the skeletal muscle subtype. Promising in this regard is the observation that a W8E replacement in KIIIA produces a 200-fold decrease in activity against Na_v1.4 [5]. In addition, recent work of Stevens and colleagues [31] showed that chimeric analogues of KIIIA and another μ -conotoxin, BuIIIC, varied in their selectivities for different subtypes of sodium channels. Our work and the recent SAR studies on KIIIA [4, 5, 31] establish the groundwork for next generation KIIIA analogues with improved selectivity profiles and efficacies. For example, exploring combinations of peptoid analogues of KIIIA in position 7 in the context of mutations at Trp⁸ or Arg¹⁴ may lead to favorable selectivity profiles for future KIIIA-derived drug leads. Current efforts in developing sodium-channel blocking conotoxins as analgesics are also complemented by exploring the antinociceptive properties of another conotoxin, namely μ O-MrVIB, which apparently acts by preferential block of the Na_v1.8 subtype of sodium channels [41, 42].

3. Conclusions

This study describes an application of the peptoid strategy towards disulfide-rich, neuroactive peptides, and provides the first demonstration that *N*-substituted glycine residues can be used to modulate bioactivity of conotoxins. Comparison of the folding and the activities of two different series of peptomer analogues leads us to suggest that the most effective strategy for lead optimization of disulfide-rich peptomers is to start with the extensive SAR studies using the disulfide-depleted selenopeptide analogues, followed by confirming the results and refining the most promising leads using peptides with a three-disulfide-bridged core.

4. Experimental Section

4.1. Chemical synthesis

Three disulfide bridged conopeptoid KIIIA analogues and *N*-terminal part of disulfide-deficient selenoconopeptoid KIIIA analogues (Fmoc-AUNCSSX-, where residue X was either Sar, Nnle or Noct) were synthesized manually by a solid-phase method by using *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry. The Fmoc-selenocysteine with the selenium-*p*-methoxybenzyl protection was purchased from ChemImpex International, (Wood Dale, IL). Peptide fragment of Fmoc-WARDHSRUC-NH₂ was obtained using peptide synthesizer (Applied Biosystems, USA). Analogues with *N*-substituted glycine derivatives were introduced into the peptide chain by the submonomeric approach [16]. In the first step the method required using of bromoacetic or chloroacetic acid activated by *N*, *N'*-diisopropylcarbodiimide (DIPCDI), while in the second step the appropriate amine derivative (butylamine or octylamine) was applied. After the last acylation step, the *N*-terminal Fmoc-residue was deprotected, the resin was rinsed with CH₂Cl₂ and dried *in vacuo*. The cleavage from the resin of three disulfide bridged μ -KIIIA peptoid analogues was performed using a mixture of trifluoroacetic acid/phenol/ethanedithiol/thioanisole/H₂O, 82.5/5/2.5/5/5 by volume. The disulfide-depleted selenoconopeptoid KIIIA analogues were cleaved from the resin with modified reagent K: trifluoroacetic acid/phenol/thioanisole/water 90/2.5/7.5/5 by volume and 1.3 equivalents of 2,2'-dithiobis(5-nitropyridine) (DTNP) [43]. In the next step, the analogues were treated with 50 mM DTT (threo-1,4-dimercapto-2,3- butanediol), 100 mM Tris-HCl, 1mM EDTA, pH 7.5 at RT. After 2 hours, acetic acid (8% final concentration) was added to quench the reaction. Peptides were purified by reversed-phase RP-HPLC using semi-preparative Kromasil-100, C₈5 μ m column (8 $\times$ 250 mm) (Knauer, Germany) over a linear gradient 15 - 90% or 20 - 90% of solvent B in 30 min. HPLC solvents were as follows: solvent (A) 0.1% TFA (v/v) in water, solvent (B) 0.1% TFA (v/v) in 80% aqueous acetonitrile. The HPLC separations were monitored at 226 nm and the flow rate was 2.5 mL/min. Oxidative folding reactions of the KIIIA analogues were carried out in the presence of 100 mM Tris-HCl, 1mM EDTA, pH 7.5 with 1 mM GSSG and 1mM GSH, at room temperature. Final peptide concentration in the folding solution was 20 μ M. After 1 hour, acetic acid (8% final concentration) was added to quench the reactions. The oxidized conopeptoid analogues were purified using the same method as described above. The purity of peptidomimetics synthesized was checked on analytical Vydac C₁₈5 μ m column (4.6 $\times$ 250 mm,) and their chemical identity was confirmed by either ESI or MALDITOF mass spectrometry.

4.2. Electrophysiology assay

KIIIA analogues were tested for their ability to block the cloned mammalian sodium channel Na_v1.2 expressed in *Xenopus laevis* oocytes. These electrophysiological experiments were carried out basically as described elsewhere [1] and [39]. Briefly, a given oocyte was injected with 30 nL of rat Na_v1.2 cRNA (1.5 ng) in distilled water. Oocytes were incubated for 1 to 2 d at 16 °C in ND96 composed of: NaCl (96 mM), KCl (2 mM), CaCl₂ (1.8 mM), MgCl₂ (1 mM), and Hepes (5 mM), pH 7.5, which was supplemented with the antibiotics penicillin (100 U/mL), streptomycin (0.1 mg/mL), amikacin (0.1 mg/mL), and Septra (0.2 mg/mL). Oocytes were perfused with ND96 and two-electrode voltage-clamped while

sodium currents were acquired using a holding potential of -80 mV and stepping the membrane potential to -10 mV for 50 ms every 20 seconds. Current signals were filtered at 2 kHz, digitized at a sampling frequency of 10 kHz and leak-subtracted by a P/8 protocol using in-house software written in LabVIEW (National Instruments, Austin, TX). The oocyte was situated in a 30 μ L well of a recording chamber fabricated from Sylgard (Dow Corning, Midland MI) and exposed to toxin by applying 3 μ L of toxin at 10-times its final concentration with a pipettor and manually stirring the bath for a few seconds by gently aspirating and expelling a few μ L of the bath fluid several times with the pipettor. Toxin exposure was in a static bath to conserve peptide. Toxin-containing solution was washed out from the well by perfusion with ND96, initially at a speed of 1.5 mL/min for 20 s, then at 0.5 mL/min thereafter. All recordings were conducted at room temperature (~ 20 °C).

The observed rate constant of toxin block, k_{obs} was obtained by fitting the time course of block of the peak sodium current by 10 μ M toxin with a single exponential function. The off-rate constant, k_{off} was measured by fitting the time course of recovery following toxin washout with a single-exponential function. Fitting of curves with a single-exponential function were done with software written in LabVIEW. The on-rate constant, k_{on} was estimated assuming the equation for a bimolecular reaction between toxin and channel, $k_{\text{obs}} = k_{\text{on}} \cdot 10 \mu\text{M} + k_{\text{off}}$ [8]. The dissociation constant, K_d was obtained from the ratio $k_{\text{off}}/k_{\text{on}}$. Values are expressed as mean $\pm$ SD ($N = 3$ oocytes).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Two series of peptoid-peptide chimeras of μ -conotoxin KIIIA were synthesized.
- Oxidative folding of μ -KIIIA peptomers was simplified using selenocysteines.
- All peptomer analogues blocked mammalian $\text{Na}_v1.2$ sodium channels.
- One of the peptomer analogues presented unique pharmacological properties.

Figure 1.

Structures of peptoid monomer side-chain residues incorporated in position 7, from left: *N*-methyl, *N*-butyl and *N*-octyl group of *N*-substituted Gly (A). Amino acid sequences and disulfide/diselenide bridges of the two sets of KIIIA analogues (B). The first set of μ -conopeptoid analogues contained all three disulfide bridges (upper amino acid sequence), while in the second set of peptomer analogues first disulfide bridge was eliminated by replacing a pair of Cys residues with Ala residues, and the second disulfide bridge was replaced with a diselenide bridge (lower amino acid sequence). In both sets Lys⁷ was replaced with *N*-methylglycine, *N*-butylglycine or *N*-octylglycine (marked as X in the sequences).

Figure 2.

Chemical syntheses of KIIIA peptomer analogues. An overview of a submonomeric method (A) used in this study. First step (a) required bromoacetic acid and *N*, *N*'-diisopropylcarbodiimide (DIPCPI), while in the second step (b) butylamine or octylamine was necessary. The preparation of three disulfide bridged μ -KIIIA peptoid analogues (B) included a cleavage from the resin using a mixture of trifluoroacetic acid/phenol/ethanedithiol/thioanisole/ H_2O , 90/7.5/2.5/5 by volume (d) followed by the oxidative folding in the presence of 1mM GSSG and 1 mM GSH in 100mM Tris-HCl, 1mM EDTA, pH 7.5

(e). The disulfide-depleted selenoconopeptoid KIIIA analogues (C) were cleaved from the resin with modified reagent K: trifluoroacetic acid/phenol/thioanisole/water 90/7.5/2.5/5 by volume and 1.3 equivalents of DTNP to remove Mob group from the selenium atom. The crude material was treated with 50 mM DTT, 100 mM Tris-HCl, 1mM EDTA, pH 7.5 (f), followed by HPLC purification and the oxidation step (e).

Figure 3.

HPLC profiles of reduced and oxidized three disulfide-bridged (A) and disulfide-deficient selenoconopeptoid KIIIA analogues (B). The oxidative folding was performed in the presence of 1 mM oxidized and 1 mM reduced glutathione and 0.1 M Tris-HCl, pH 7.5, at room temperature. The folding mixtures were quenched after 1 hr by an addition of 8% formic acid and separated on semi-preparative C₈ HPLC column. The biologically-active oxidation product for each three disulfide-bridged KIIIA analogue on the Na_v1.2 sodium channel is indicated with an asterisk.

Figure 4.

Activity of [Sar⁷]KIHA (10 μ M) on Na_v1.2 expressed in oocytes. Oocytes were voltage clamped, and voltage-gated sodium currents (I_{Na}) were recorded as described in the Experimental Section. Representative currents traces of Na_v1.2 before (gray trace) and during exposure to 10 μ M [Sar⁷]KIHA (black trace) (A). Representative time courses of block by 10 μ M [Sar⁷]KIHA and recovery during washout. Black bar above plot indicated

presence of 10 μM [Sar⁷]KIIIA (B).). Kinetic constant values of block and recovery in replicate experiments are summarized in Table 2.

Table 1

Physicochemical properties of KIIIA analogues

Peptide	HPLC (t_R) [min] ^a	Calculated molecular mass [M ⁺] _{calc}	Experimental molecular mass [M+H ⁺] _{exp} or [M ⁺] _{exp}
[Sar ⁷]KIIIA	9,4	1825,17	1826,3
[Ala ^{1,9} ,Sec ^{2,15} ,Sar ⁷]KIIIA	9,5	1860,1	1860,3*
[Nnle ⁷]KIIIA	12,5	1868,16	1868,6
[Ala ^{1,9} ,Sec ^{2,15} ,Nnle ⁷]KIIIA	12,4	1902,3	1902,2*
[Noct ⁷]KIIIA	18,3	1923,27	1924,1
[Ala ^{1,9} ,Sec ^{2,15} ,Noct ⁷]KIIIA	18,4	1958,9	1958,4*

^aHPLC was performed using Waters (USA) system, Vydac C18 column (4.6 × 250 mm, 5-μm particle size), solvent system: (A) 0,1% TFA, (B) 90% ACN in A; linear gradient from 5 to 65% B in 30 min, flow rate 1ml/min., monitored at 220 nm;

Table 2Kinetics and affinities of the peptomer analogues in blocking $\text{Na}_v1.2$ expressed in *Xenopus* oocytes

Peptide	% block (10 μM)	K_{obs} (min^{-1})	K_{off} (min^{-1})	Estimated K_{on} ($\mu\text{M}^{-1}\text{min}^{-1}$)	K_d (μM)
KIIIA	90 $\pm$ 3 ^a	0.36 $\pm$ 0.06 ^a	0.0016 $\pm$ 0.00016 ^b	0.3 $\pm$ 0.03 ^b	0.0053 $\pm$ 0.0005 ^b
[Sar ⁷]KIIIA	69 $\pm$ 4	1.1 $\pm$ 0.5	0.006 $\pm$ 0.001	0.11 $\pm$ 0.05	0.055 $\pm$ 0.03
[Ala ^{1,9} ,Sec ^{2,15} ,Sar ⁷]KIIIA	70 $\pm$ 3	3.8 $\pm$ 0.7	0.35 $\pm$ 0.13	0.35 $\pm$ 0.071	1 $\pm$ 0.4
[Nhle ⁷]KIIIA	82 $\pm$ 4	0.36 $\pm$ 0.07	0.01 $\pm$ 0.003	0.035 $\pm$ 0.007	0.29 $\pm$ 0.1
[Ala ^{1,9} ,Sec ^{2,15} ,Nhle ⁷]KIIIA	85 $\pm$ 3	1.9 $\pm$ 0.5	0.23 $\pm$ 0.06	0.17 $\pm$ 0.05	1.4 $\pm$ 0.5
[Noct ⁷]KIIIA	80 $\pm$ 1	0.26 $\pm$ 0.04	0.01 $\pm$ 0.002	0.025 $\pm$ 0.004	0.4 $\pm$ 0.1
[Ala ^{1,9} ,Sec ^{2,15} ,Noct ⁷]KIIIA	81 $\pm$ 3	4.4 $\pm$ 0.95	0.21 $\pm$ 0.07	0.42 $\pm$ 0.1	0.5 $\pm$ 0.2

^aData from reference [1].^bdata from reference [2]. Values $\pm$ SE from at least three different oocytes.