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**Solution structure and functional analysis of HelaTx1: the first toxin member of the  $\kappa$ -KTx5 subfamily**

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Structure information: NMR assignment data and atomic coordinates for HelaTx1 have been deposited in the Protein Data Bank (entry 2NDD).

*Key words:* HelaTx1; voltage-gated potassium channels; scorpion toxin; structural topology; alanine-scanning analog

**Abstract**

Scorpion venom comprises a cocktail of toxins that have proven to be useful molecular tools for studying the pharmacological properties of membrane ion channels. HelaTx1, a short peptide neurotoxin isolated recently from the venom of the scorpion *Heterometrus laoticus*, is a 25 amino acid peptide with two disulfide bonds that shares low sequence homology with other scorpion toxins. HelaTx1 effectively decreases the amplitude of the K<sup>+</sup> currents of voltage-gated Kv1.1 and Kv1.6 channels expressed in *Xenopus* oocytes, and was identified as the first toxin member of the  $\kappa$ -KTx5 subfamily, based on a sequence comparison and phylogenetic analysis. In the present study, we report the NMR solution structure of HelaTx1, and the major interaction points for its binding to voltage-gated Kv1.1 channels. The NMR results indicate that HelaTx1 adopts a helix-loop-helix fold linked by two disulfide bonds without any  $\beta$ -sheets, resembling the molecular folding of other cysteine-stabilized helix-loop-helix (Cs  $\alpha/\alpha$ ) scorpion toxins such as  $\kappa$ -hefutoxin, HeTx, and OmTx, as well as conotoxin p114a. A series of alanine-scanning analogs revealed a broad surface on the toxin molecule largely comprising positively-charged residues that is crucial for interaction with voltage-gated Kv1.1 channels. Interestingly, the functional dyad, a key molecular determinant for activity against voltage-gated potassium channels in other toxins, is not present in HelaTx1.

*Abbreviations:* CD, circular dichroism; HefuTx1,  $\kappa$ -hefutoxin 1; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear overhauser effect spectroscopy; NOE, nuclear overhauser effect; PDB, protein data bank.

## 1. Introduction

Envenomation from poisonous animals usually results in pain, paresthesia, and swelling, attributed mainly to marked over-activity of the autonomic nervous system [1, 2]. Scorpion venom contains various toxins that block membrane ion channels responsible for either sodium, potassium, calcium or chloride membrane permeation [3-5]. These scorpion toxins are classified into several families according to target channel type, the structural topology of toxins, and the type of response induced [3-5]. Among them, scorpion toxins affecting K<sup>+</sup> channels (KTxs) are divided into four families ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\kappa$ -KTxs) based on the length of the amino acid sequence and the number of cysteine residues present [3, 5-7]. Additionally,  $\kappa$ -type toxins are further subdivided into  $\kappa$ -KTx1, 2, 3, 4, and 5 subtypes based on K<sup>+</sup> channel subtype specificity [5, 6, 8]. These diverse KTxs are useful tools for structural and functional characterization of K<sup>+</sup> channels, and many studies have investigated interactions between KTxs and the surfaces of channels, providing insight for the rational design of potential lead compounds and therapeutic drug candidates to treat ion channel-related diseases [6].

HelaTx1, a peptide neurotoxin isolated recently from the venom of the scorpion *Heterometrus laoticus*, consists of 25 amino acid residues with two disulfide bonds, and is the first identified toxin member of the  $\kappa$ -KTx5 subfamily, most active against voltage-gated Kv1.1 and Kv1.6 channels [8]. Many scorpion toxins have a key molecular determinant, termed the functional dyad, that acts on voltage-gated potassium channels [7, 9]. This functional dyad, consisting of a basic lysine and an aromatic amino acid (mostly Phe or Tyr), has been widely used as a working concept to explain how peptide toxins are able to specifically interact with their targeting ion channels. Although HelaTx1 interacts specifically with voltage-gated Kv1-type channels, the key aromatic amino acid of the functional dyad is not present, and there are numerous basic residues in its primary sequence. Interestingly, it was previously reported that a single amino acid (Ser1) at the N-terminal end of HelaTx1 is more important for exerting its

blocking activity than the C-terminal hexapeptide (Asn20–His25), hence a functional role for the basic central region constrained by disulfide bonds remains to be proven [10].

In the present study, we report the NMR solution structure of HelaTx1, together with an in-depth structure-activity relationship analysis of this unique scorpion toxin. To address the structural and functional contributions of the cysteine-rich central region, we synthesized a series of alanine-scanning analogs of HelaTx1(1–19) and examined their inhibitory activities against voltage-gated Kv1.1 channels. The results suggest a unique binding mode as an alternative for the functional dyad in the interaction of the peptide with voltage-gated potassium channels.

## 2. Results and Discussion

### 2.1. Synthesis of HelaTx1 and its analogs

Synthesis and oxidative folding of the linear precursor of HelaTx1 resulted in a single major peak in analytical HPLC. MALDI-TOF-MS analysis of the HelaTx1 yielded the expected molecular mass (2917.2 Da) in good agreement with the theoretical value. Similarly, The synthesis and oxidative folding of HelaTx1(1–19) and its alanine-scanning analogs (residues replaced with alanine, except for cysteine residues) were straightforward, suggesting that deletion of the C-terminal part (Asn20 to His25) of HelaTx1 and amino acid replacement in the cysteine-rich region did not affect the molecular folding of the peptides [10].

It was previously reported that trypsin digestion of HelaTx1 gave two major fragments: one with a Cys2-Cys19 disulfide bond and another with a Cys6-Cys15 combination [8, 10]. Enzymatic fragmentation by trypsin or chymotrypsin digestion showed that all synthetic alanine scanning analogs possessed the same disulfide bond combination as native HelaTx1. As shown in Fig. 1A, CD spectra for wild-type HelaTx1, HelaTx1(1–19), and its four analogs (S1A, S9A, K13A, and K14A) are quite similar to that of the parental HelaTx1, indicating that

HelaTx1 and its analogs have a similar overall fold, with conserved disulfide connectivity.

## 2.2. NMR analysis and structural calculation

To better understand the structure-activity relationships of HelaTx1, solution structures of HelaTx1 were determined using traditional two-dimensional NMR technique. Sequence-specific resonance assignments were established according to a standard protocol. Identification of the amino acid spin system was based on scalar coupling patterns observed in DQF-COSY and TOCSY experiments, complemented by the results of NOESY measurements. The identified spin system was ordered along the primary structure of HelaTx1 through inter-residue NOEs observed on the NOESY spectrum. The pattern of observed NOEs was ultimately interpreted in terms of the secondary structure of the HelaTx1 molecule. It is likely that the HelaTx1 toxin is mainly organized into helical and long loop regions, based on the presence of short- and medium-range NOEs, small J-coupling constants, and slowly exchanging amide protons.

For structure calculation, we used 257 distance constraints derived from inter-proton NOE peaks, eight dihedral angle constraints from coupling constants, six hydrogen bond constraints, and six disulfide bond constraints, giving a total of 277 constraints. Using these constraints, we carried out simulated annealing calculations starting from 100 initial random HelaTx1 structures, and selected 20 final structures that were in good agreement with the NMR experimental constraints, for which NOE distance and torsion angle violations were  $<0.5 \text{ \AA}$  and  $<5^\circ$ , respectively. The low values for van der Waals energy and the small deviations from idealized covalent geometry indicate the absence of serious bad contacts and distortions in the converged structures. Average RMS deviations calculated for backbone heavy atoms (N, C $\alpha$ , and C) and all heavy atoms in residues 2–23 were  $0.99 \pm 0.25 \text{ \AA}$  and  $2.03 \pm 0.29 \text{ \AA}$ , respectively. Ramachandran analysis using the program PROCHECK-NMR revealed that backbone

dihedral angles for all residues in the 20 final structures fell either in most favored regions (76.2%) or in generously allowed regions (23.8%). On the basis of these results, it is likely that the HelaTx1 molecule is relatively highly flexible due to the inherent flexibility of the N-terminal helical region and a long loop region between helix I and helix II, consistent with the lack of medium- and long-range NOE constraints from both regions. Fig. 1B shows the best-fit superposition of the 20 converged structures. Overall, the solution structure of HelaTx1 (PDB: 2NDD) consists of two parallel helices composed of residues Lys3–Cys6 (helix I) and Cys15–Gly24 (helix II), which are held in position by two disulfide bridges, Cys2–Cys19 and Cys6–Cys15, and a long loop between helix I and helix II, without any  $\beta$ -sheets (Fig. 1C).

### 2.3. Surface profile of HelaTx1

As an approach to understand the specific mode of interaction between peptide toxins and ion channels, it is very useful to determine the degree of solvent exposure for each amino acid residue in the toxin molecule. To this end, the solvent-accessible surface profile of the HelaTx1 structure was analyzed, categorizing each residue as either buried or exposed. From this analysis, more than half of the HelaTx1 amino acids (~70%) appeared to be exposed to solvent, with the exception of cysteine residues forming an intramolecular disulfide bond. As would be expected, the majority of solvent-exposed amino acids are hydrophilic. As shown in Fig. 2A and 2B, the surface regions exposed to the external environment are composed mainly of positively- (~60%; Lys3, Lys4, Arg10, Arg11, Lys13, Lys14, Lys18, Arg21, His23, and His25) and negatively- (~10%; Asp5 and Asp22) charged amino acids. By contrast, other  $\kappa$ -KTx family members, such as HefuTx1 and OmTx1, are composed of positively- (~35.7% in HefuTx1 and ~23% in OmTx1) and negatively- (~29% in HefuTx1 and ~24% in OmTx1) charged residues.

As mentioned previously, HelaTx1 adopts a three-dimensional structure that consists of

an N-terminal short helix (helix I) and a C-terminal long helix (helix II), connected by a long loop between helix I and helix II. Interestingly, most of the basic residues are relatively equally distributed in a broad region over the entire molecule from the N-terminus to the C-terminus, and are not clustered on one side of the molecule (Fig. 2A). It is noteworthy that the HelaTx1 molecule does not contain any aromatic amino acids (Phe or Tyr), and hence lacks a key determinant that forms the functional dyad together with a lysine residue in related peptide toxins.

#### 2.4. Biological assays of HelaTx1 and its analogs

HelaTx1, a 25-mer peptide toxin, is rich in positively-charged amino acids. It lacks the sequence characteristics of a functional dyad, and belongs to the fifth subfamily of the  $\kappa$ -scorpion toxins that act on voltage-gated potassium channels [3, 8]. Competition experiments with tetraethylammonium showed that the toxin acts as a pore blocker [8, 11]. Previously, we reported that HelaTx1(1–19) and HelaTx1(2–19) inhibit voltage-gated Kv1.1 channels with about three-fold and five-fold less potency than wild-type HelaTx1, respectively, indicating that only a single amino acid (Ser1) at the N-terminus is relatively more important for functional activity than the C-terminal hexapeptide (Asn20–His25). The  $IC_{50}$  value was  $0.579 \pm 0.094 \mu\text{M}$  for HelaTx1,  $1.937 \pm 0.34 \mu\text{M}$  for HelaTx1(1–19), and  $3.111 \pm 0.128 \mu\text{M}$  for HelaTx1(2–19) [10]. To further assess the structure-activity relationships of the toxin, HelaTx1, HelaTx1(1–19) and HelaTx1(2–19) analogs were synthesized in which amino acids at each position were substituted by alanine. Although there may be local structural differences around replaced residues, we assumed that alanine replacement did not affect the overall conformation of HelaTx1, based on their CD spectra (Fig. 1A). The inhibitory effects of synthetic HelaTx1, HelaTx1(1–19) and HelaTx1(2–19) alanine-scanning analogs were tested using Kv1.1 channels, the most sensitive to HelaTx1 [10]. Concentration-dependent inhibitory curves and

IC<sub>50</sub> values were obtained for all synthetic peptides (Fig. 3A). As shown in Fig. 3A and 3B, alanine replacement of both Lys13 and Lys14 resulted in a marked loss of inhibitory activity, and alanine replacement of other basic residues (Lys3, Lys4, Arg10, Arg11, and Lys18) and Gly8 also reduced the inhibitory activity significantly, equating to a >10-fold reduction in potency. Four functionally important basic residues (Arg10, Arg11, Lys13, and Lys14) are partially clustered on the edge of a long loop region between helix I and helix II (Fig. 3C). Interestingly, alanine replacement of Ser1 or Ser9 caused a slight enhancement in activity, while all other alanine replacements except for Glu5 reduced the inhibitory effect on Kv1.1 channels. The series of alanine-scanning analogs revealed that a broad surface composed in large part by positively-charged residues is crucial for interaction with Kv1.1 channels, and demonstrated that a functional dyad is not the sole determinant in the interaction with this ion channel.

### 2.5. Structural comparison between *HelaTx1* and other toxins

Peptide toxin blockers interacting with voltage-gated potassium channels can be classified into cysteine-stabilized  $\alpha$ -helix/ $\beta$ -sheet (CS  $\alpha/\beta$ ), cysteine-stabilized  $\alpha$ -helix-loop- $\alpha$ -helix (CS  $\alpha/\alpha$ ), and inhibitor cysteine knot (ICK) groups according to their three-dimensional structures [5, 12]. The solution structure of *HelaTx1* used in this study adopts the CS  $\alpha/\alpha$  fold with a helix-loop-helix linked by the two disulfide bonds, and shares this fold with other scorpion toxins including HefuTx1 from the scorpion *Heterometrus fulvipes*,  $\kappa$ -KTx1.3 from *Heterometrus spinifer*, OmTx1 from *Opisthacanthus madagascarienses*, HeTx203 and HeTx204 from scorpion cDNA libraries, and p114a from *Conus planorbis* venom (Fig. 4) [13-16]. A number of peptides with this fold have been identified in venom from scorpions and cone snails [3, 7, 17, 18]. It is noteworthy that the CS  $\alpha/\alpha$  fold is an evolutionarily conserved

structural motif shared by a large group of polypeptides acting as functional modulators against various membrane ion channels.

HefuTx1, the first  $\kappa$ -KTx to be described that adopts the CS  $\alpha/\alpha$  scaffold, effectively blocks both Kv1.2 and Kv1.3 channels at micromolar levels, while OmTx1 differentially inhibits Kv1.1, Kv1.2, and Kv1.3 channels, and HeTx204 is most sensitive toward Kv1.3 and KCNQ1 channels. HelaTx1 used in this study effectively decreases the amplitude of the K<sup>+</sup> currents of the Kv1.1 and Kv1.6 channels. Although all of these toxins share the same molecular topology, their pharmacological effects against Kv1-type channels differ. Based on sequence comparison and phylogenetic analysis, HefuTx1 was classified as a toxin member of  $\kappa$ -KTx1 subfamily, OmTx1 and HeTx are members of the  $\kappa$ -KTx2 subfamily, and HelaTx1 is the first toxin member of the  $\kappa$ -KTx5 subfamily [8].

HefuTx1 specifically interacts with Kv1-type channels through a so-called functional dyad, consisting of a hydrophobic residue and a lysine residue (Tyr5 and Lys19), which is fully exposed from a flat surface formed by the edge of the two parallel helices [13]. Interestingly, this functional dyad is conserved in many other toxins targeting voltage-gated potassium channels, such as charybdotoxin, hanatoxin, and  $\kappa$ -conotoxin, and is used as a working concept to explain how toxins are able to recognize and block their specific ion channels [16, 17, 19, 20]. Although HelaTx1 effectively blocks voltage-gated Kv1-type channels, the functionally important site of the toxin molecule is composed of a number of basic residues without an aromatic amino acid, and thus lacks a key factor of the functional dyad, indicating that the mode of action of HelaTx1 differs from that of HefuTx1 [21]. Unlike other peptide toxins, the molecular surface of HelaTx1 is highly enriched in positively-charged basic residues (Lys3, Lys4, Gly8, Arg10, Arg11, Lys13, Lys14, and Lys18), which are functionally important and broadly distributed over the entire molecule. These results may indicate a unique binding mode involving an intimate interaction between negatively-charged channel residues and positively-

charged toxin residues. Interestingly, although acidic scorpion toxins (OmTx and HeTx) and conotoxin pl14a share very low sequence homology with HelaTx1, except for cysteine residues, they share a similar structural topology and functional ability to block Kv1-type channels (Fig. 4) [13-15].

In summary, previous structure-activity relationship studies on various scorpion toxins that act on voltage-gated K<sup>+</sup> channels suggest that a pair of well-defined basic and aromatic residues, referred to as the functional dyad, plays a key role in toxin action toward these channels. Herein, we determined the three-dimensional structure of HelaTx1, which adopts a helix-loop-helix tertiary structure, and we examined the relative contribution of each amino acid in HelaTx1 to toxin action against voltage-gated Kv1.1 channels. Functional characterization showed that both Lys13 and Lys14 are essential for inhibition of Kv1.1 channel activity (Fig. 3). In addition, residues Lys3, Lys4, Gly8, Arg10, Arg11, and Lys18 are also important for activity. Many of the basic residues essential for HelaTx1 activity are broadly distributed over the entire molecule from the N-terminal to the C-terminal regions, and there is a distinct basic cluster on the edge of the loop region that connects the two helices. Our results indicate that the integrity of the functional dyad is not a full prerequisite for toxin action on Kv1.1 channels, suggesting a unique binding mode as an alternative for the functional dyad in the interaction between peptide toxins and Kv1-type channels. HelaTx1 can thus be considered an interesting lead compound for the design of novel compounds targeting voltage-gated Kv-type channels.

### 3. Materials and Methods

#### 3.1. Peptide synthesis

For synthesis of HelaTx1, HelaTx1 (1-19), and other HelaTx1 (1-19) analogs, Solid-phase peptide synthesis was performed using solid-phase Fmoc chemistry as previously

described [10]. The peptides were purified by RP-HPLC and confirmed by MALDI-TOF-MS measurements.

### 3.2. CD and NMR experiments

CD spectra were recorded on a J-1100 spectropolarimeter (JASCO, Tokyo, Japan). The results are expressed as molar ellipticity  $[\theta]$ . Spectra were measured in wavelength ranging from 190 to 250 nm.

NMR spectra for HelaTx1 were recorded on a Bruker DMX 600 MHz spectrometer (Bruker, Billerica, USA). Peak assignments were determined using traditional two-dimensional NMR sequential assignment techniques. Ambiguities in the assignments due to peak overlap were resolved by comparison of NOESY and TOCSY spectra, and through the use of NOE constraints obtained at two different temperatures (288 K and 298 K). Slowly exchanging amide protons were identified in a series of TOCSY experiments with time scales of 2.5, 5, 7.5, 10, 12.5, 15, and 24 h.

### 3.3. Structure calculations

A set of 20 energy-minimized stable structures were chosen as the low-energy structures among a total of 100 structures. The structure of the peptide was calculated based on distance constraints derived from NOESY spectra using X-PLOR version 3.1 [22]. Backbone dihedral angle restraints derived from  $^3J_{\text{NH}\alpha\text{H}}$  coupling constants were measured from a one-dimensional spectrum or from the DQF-COSY spectrum. We observed RMS deviations from the experimental distance and dihedral constraints, from the energetic statistics, and from the idealized geometry. Validation of the final structures was accomplished using PROCHECK-NMR. Structures were analyzed using MOLMOL and PYMOL. The distributions of backbone dihedral angles in the final converged structure were evaluated by representation of the

Ramachandran dihedral pattern.

### 3.4. Expression in *Xenopus* oocytes

For expression of rKv1.1 channels in *Xenopus* oocytes, linearized plasmids were transcribed using a T7 mMESSAGE-mMACHINE transcription kit (Ambion, Texas, USA). Stage V–VI oocytes were harvested from an anesthetized *Xenopus laevis* frog as previously described [23, 24]. Oocytes were then injected with 50 nl of cRNA at a concentration of 1 ng/nl using a micro-injector (Drummond Scientific, Broomall, USA).

### 3.5. Electrophysiological recordings

Two-electrode voltage-clamp recordings were performed by using a Geneclamp 500 amplifier (Molecular Devices, San Jose, USA) controlled by a pClamp data acquisition system (Axon Instruments, Foster City, USA). Whole-cell currents were recorded from oocytes 1–4 days after injection. To determine the current-voltage relationship, currents were evoked by depolarizing cells from -90 mV to +50 mV in a series of 10 mV increments. All data represent at least three independent experiments ( $n \geq 3$ ) and are presented as the mean  $\pm$  standard error.

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### Figure legends

Fig. 1. (A) CD spectra for HelaTx1, HelaTx1 (1–19), and HelaTx1 (1–19) analogs expressed in terms of molar ellipticity  $[\theta]$ . (B) Superposition of the 20 lowest energy HelaTx1 structures. (C) Ribbon representation of the average structure of HelaTx1 (PDB: 2NDD).

Fig. 2. Molecular surface of hydrophilic residues in HelaTx1. (A) Ribbon representation and (B) surface profile colored according to electrostatic potential (basic and acidic residues are colored blue and red, respectively).

Fig. 3. (A) Dose-response curves for HelaTx1, HelaTx1 (1–19), and HelaTx1 (1–19) analogs. (B)  $IC_{50}$  values for HelaTx1 (1–19) and HelaTx1 (1–19) analogs against the Kv1.1 channel. The dotted line indicates the  $IC_{50}$  value of HelaTx1 (1–19). (C) Representative  $K^+$  currents showing the inhibitory effect of HelaTx1, HelaTx1 (1–19), and HelaTx1 (1–19) analogs (R10A, R11A, K13A, and K14A).

Fig. 4. Comparison of the surface profiles of HelaTx1, other  $\kappa$ -KTX family members, and conotoxin p114a. Note the conserved structural motif with a CS  $\alpha/\alpha$  fold. Ribbon and surface representations of (A) HelaTx1, (B) HefuTx1, (C) OmTx1, and (D) p114a are shown. Amino acid sequences of these toxins are shown at the bottom of the figure. Hydrophobic residues are colored green, and basic and acidic residues are colored by blue and red, respectively.

### References

1. Gwee MC, Nirathanan S, Khoo HE, Gopalakrishnakone P, Kini RM & Cheah LS (2002) Autonomic effects of some scorpion venoms and toxins, *Clin Exp Pharmacol Physiol.* **29**, 795-801.
2. Chandy KG, Wulff H, Beeton C, Pennington M, Gutman GA & Cahalan MD (2004)  $K^+$

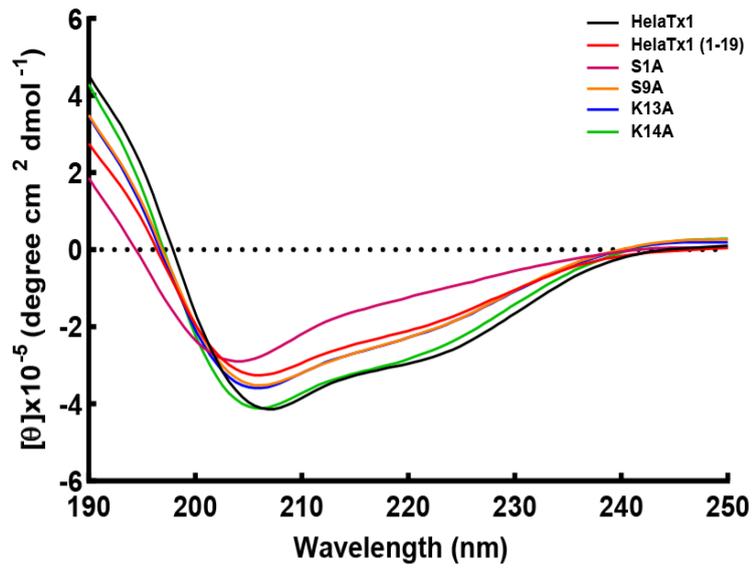
channels as targets for specific immunomodulation, *Trends Pharmacol Sci.* **25**, 280-9.

3. Rodriguez de la Vega RC & Possani LD (2004) Current views on scorpion toxins specific for K<sup>+</sup>-channels, *Toxicon.* **43**, 865-75.
4. Rodriguez de la Vega RC & Possani LD (2005) Overview of scorpion toxins specific for Na<sup>+</sup> channels and related peptides: biodiversity, structure-function relationships and evolution, *Toxicon.* **46**, 831-44.
5. Quintero-Hernandez V, Jimenez-Vargas JM, Gurrola GB, Valdivia HH & Possani LD (2013) Scorpion venom components that affect ion-channels function, *Toxicon.* **76**, 328-42.
6. Ortiz E, Gurrola GB, Schwartz EF & Possani LD (2015) Scorpion venom components as potential candidates for drug development, *Toxicon.* **93**, 125-35.
7. Zhu S, Peigneur S, Gao B, Luo L, Jin D, Zhao Y & Tytgat J (2011) Molecular diversity and functional evolution of scorpion potassium channel toxins, *Mol Cell Proteomics.* **10**, M110 002832.
8. Vandendriessche T, Kopljar I, Jenkins DP et al (2012) Purification, molecular cloning and functional characterization of HelaTx1 (*Heterometrus laoticus*): the first member of a new kappa-KTX subfamily, *Biochem Pharmacol.* **83**, 1307-17.
9. Mouhat S, De Waard M & Sabatier JM (2005) Contribution of the functional dyad of animal toxins acting on voltage-gated Kv1-type channels, *J Pept Sci.* **11**, 65-8.
10. Peigneur S, Esaki N, Yamaguchi Y, Tytgat J & Sato K (2016) Effects of deletion and insertion of amino acids on the activity of HelaTx1, a scorpion toxin on potassium channels, *Toxicon.* **111**, 1-5.
11. Al-Sabi A, Shamotienko O, Dhochartaigh SN et al (2010) Arrangement of Kv1 alpha subunits dictates sensitivity to tetraethylammonium, *J Gen Physiol.* **136**, 273-82.
12. Gao B, Harvey PJ, Craik DJ, Ronjat M, De Waard M & Zhu S (2013) Functional evolution of scorpion venom peptides with an inhibitor cystine knot fold, *Biosci Rep.* **33**.

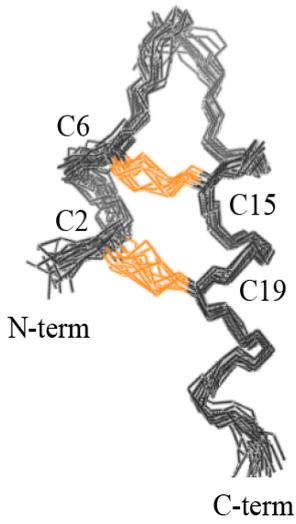
13. Srinivasan KN, Sivaraja V, Huys I et al (2002) kappa-Hefutoxin1, a novel toxin from the scorpion *Heterometrus fulvipes* with unique structure and function. Importance of the functional diad in potassium channel selectivity, *J Biol Chem.* **277**, 30040-7.
14. Chagot B, Pimentel C, Dai L et al (2005) An unusual fold for potassium channel blockers: NMR structure of three toxins from the scorpion *Opisthacanthus madagascariensis*, *Biochem J.* **388**, 263-71.
15. Chen ZY, Zeng DY, Hu YT et al (2012) Structural and functional diversity of acidic scorpion potassium channel toxins, *PLoS One.* **7**, e35154.
16. Imperial JS, Bansal PS, Alewood PF et al (2006) A novel conotoxin inhibitor of Kv1.6 channel and nAChR subtypes defines a new superfamily of conotoxins, *Biochemistry.* **45**, 8331-40.
17. Mouhat S, Jouirou B, Mosbah A, De Waard M & Sabatier JM (2004) Diversity of folds in animal toxins acting on ion channels, *Biochem J.* **378**, 717-26.
18. Moller C, Rahmankhah S, Lauer-Fields J, Bubis J, Fields GB & Mari F (2005) A novel conotoxin framework with a helix-loop-helix (Cs alpha/alpha) fold, *Biochemistry.* **44**, 15986-96.
19. MacKinnon R, Reinhart PH & White MM (1988) Charybdotoxin block of Shaker K<sup>+</sup> channels suggests that different types of K<sup>+</sup> channels share common structural features, *Neuron.* **1**, 997-1001.
20. Li-Smerin Y & Swartz KJ (2000) Localization and molecular determinants of the Hanatoxin receptors on the voltage-sensing domains of a K(+) channel, *J Gen Physiol.* **115**, 673-84.
21. Mouhat S, Mosbah A, Visan V et al (2004) The 'functional' dyad of scorpion toxin Pi1 is not itself a prerequisite for toxin binding to the voltage-gated Kv1.2 potassium channels, *Biochem J.* **377**, 25-36.

22. Badger J, Kumar RA, Yip P & Szalma S (1999) New features and enhancements in the X-PLOR computer program, *Proteins*. **35**, 25-33.
23. Rasar MA & Hammes SR (2006) The physiology of the *Xenopus laevis* ovary, *Methods Mol Biol.* **322**, 17-30.
24. Abdel-Mottaleb Y, Corzo G, Martin-Eauclaire MF et al (2008) A common "hot spot" confers hERG blockade activity to alpha-scorpion toxins affecting K<sup>+</sup> channels, *Biochem Pharmacol.* **76**, 805-15.

A



B



C

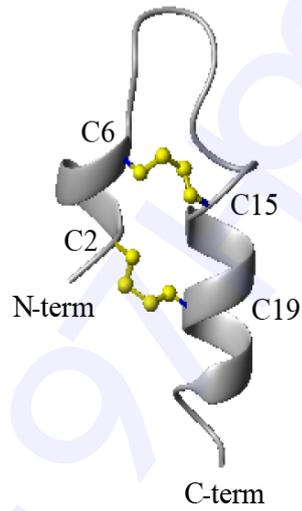
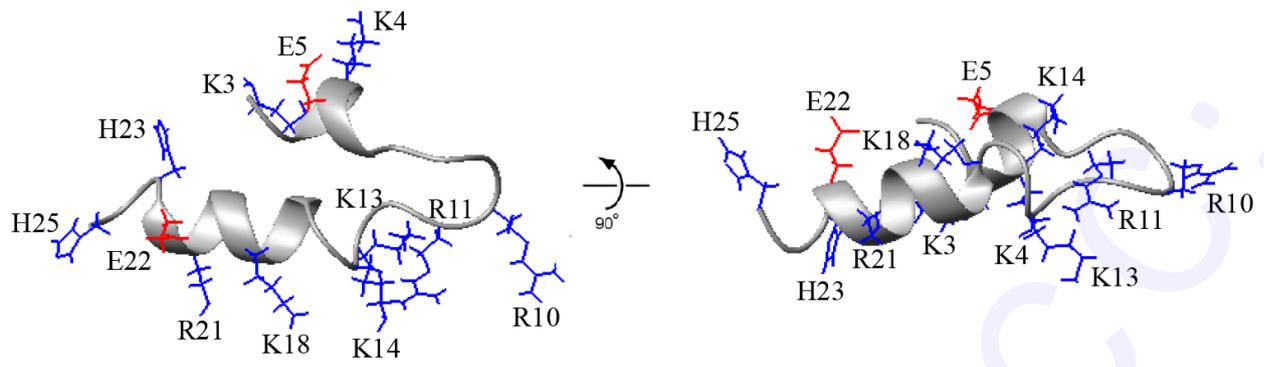


Fig. 1. FIG1

A



B

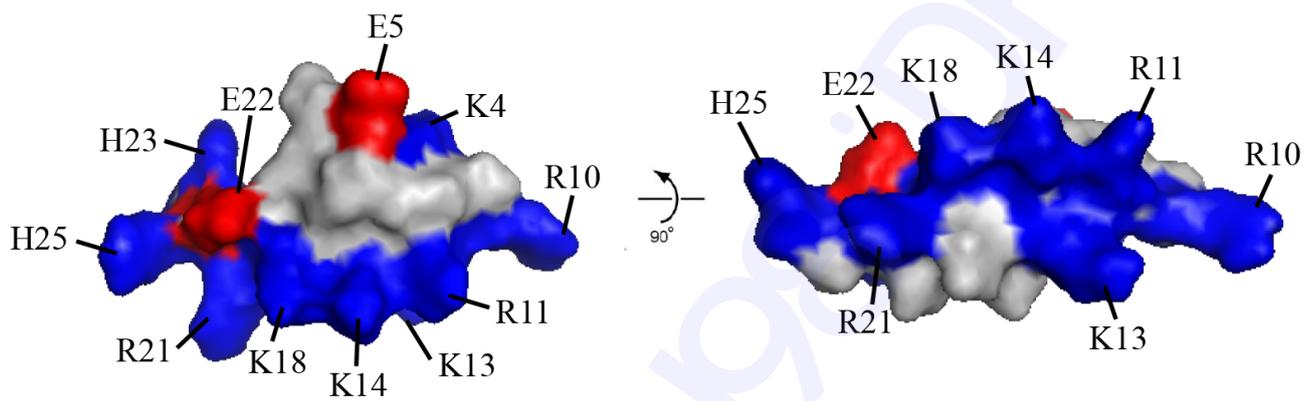


Fig. 2. FIG2

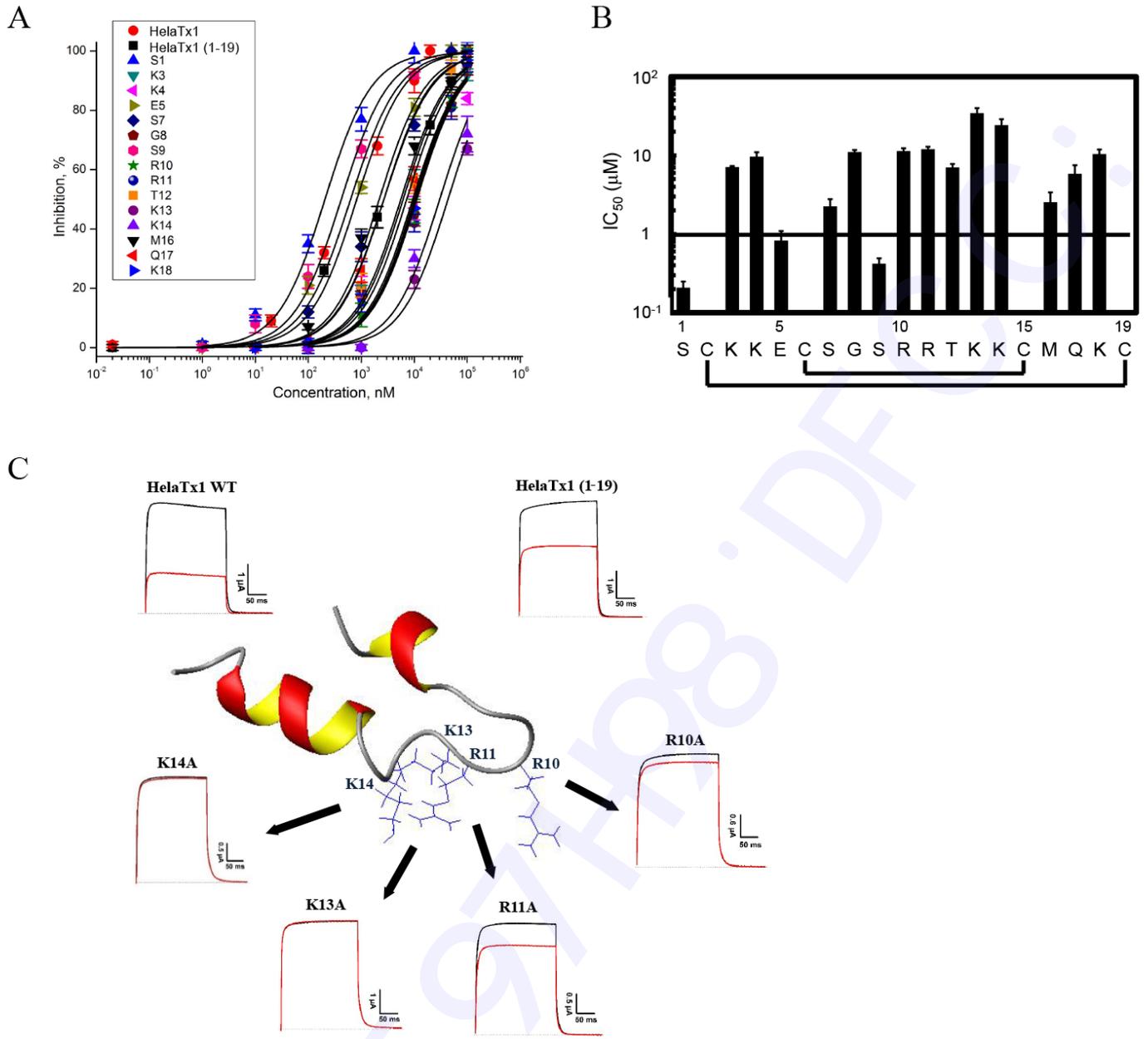


Fig. 3. FIG3

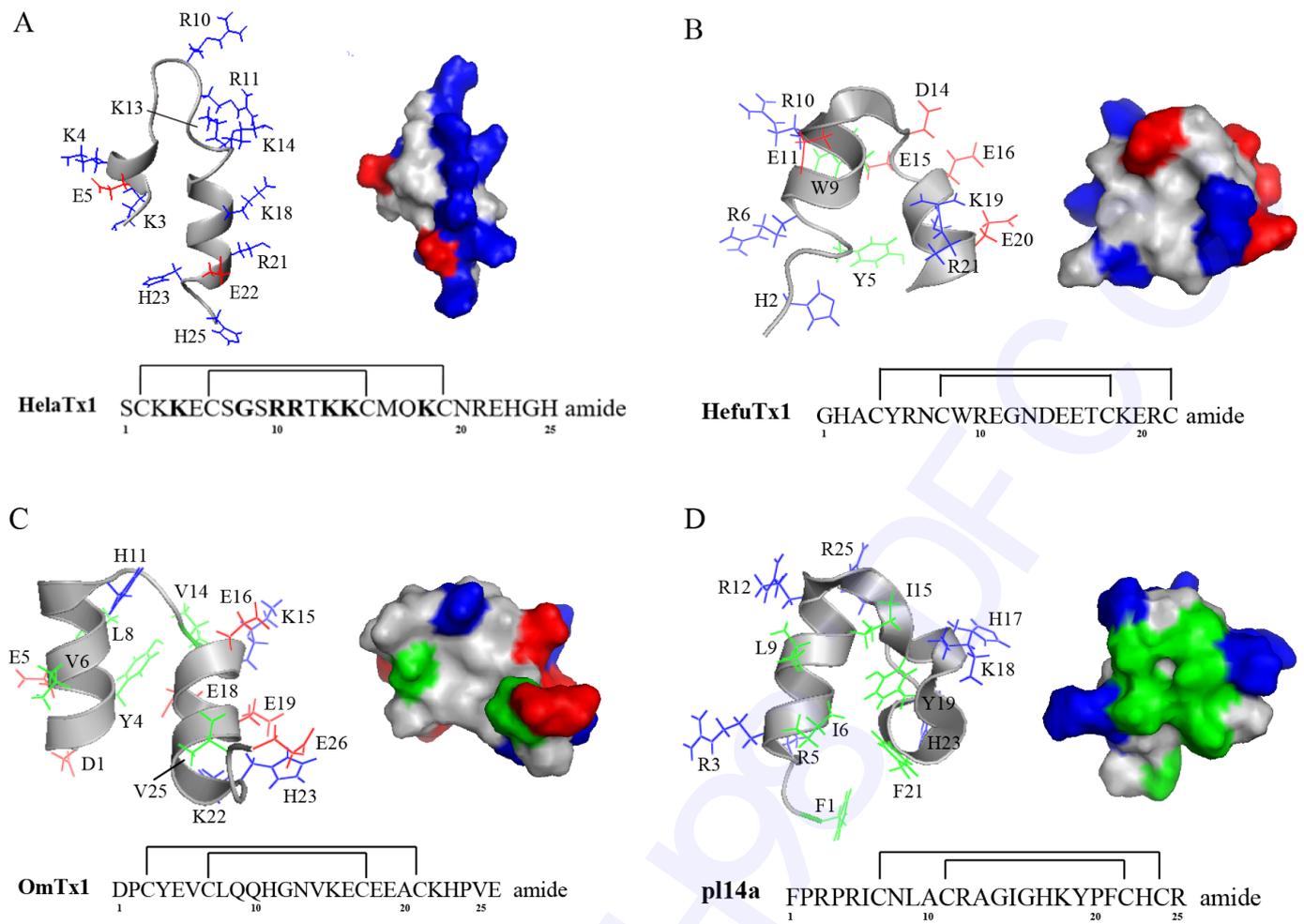


Fig. 4. FIG4