Supplemental Information for

^{19}F NMR chemical shift encoded peptide screening targeting the potassium channel Kv1.3

Supplementary information

Catalog

1.Supplementary method

1.1 Solid-phase Synthesis of HsTX1 analogies

HsTX1 analogies were synthesized by a fluoren-9-ylmethoxycarbonyl （Fmoc）-based solid-phase peptide synthesis method on an automated peptide synthesizer (CS 136XT). Peptide chains were assembled stepwise on the Rink-amide resin by repeated Fmoc deprotection and amino-coupling operations. Removal of the Fmoc group was performed twice (5, 10 min) with 20% piperidine in DMF (v/v). Coupling of Fmoc amine acids were performed twice (30, 15 min) using 4 eq Fmoc-AA-OH, 4 eq HCTU and 8 eq DIEA in DMF. The specific coupling of fluorinated amino acids was carried out manually using 2 eq Fmoc-AA-OH，2 eq HATU and 4 eq DIEA, and the reaction was performed once (1.5 h) using a constant temperature shaker at 37°C. After assembly of peptide chains, the resin was treated with cleavage cocktail K (TFA/phenol/H2O/thioanisole/1,2-ethanedithiol, 82.5:5:5:5:2.5) for 2 h. The cleavage solution was collected by filtration, condensed by blowing with N2 and precipitated with ice cold diethyl ether to give the crude peptide. The crude peptide was purified by semi-preparative RP-HPLC. The collected fractions were combined and freeze-dried. The purity of the purified peptide was confirmed by analytical RP-HPLC and ESI-MS.

The purified linear HsTX1 analogies was dissolved in redox buffer containing 1 mM GSH /0.1 mM GSSG and the pH was adjusted to 8.5 with 1 M NaOH. The folding reaction contained 0.1 mg/ml peptide and stirred under 50 r/min at 37℃. The folding process was monitored by the analytical RP-HPLC and reached equilibrium in 4 h. The molecular weight of the main peak is 8 Da less than the corresponding linear polypeptide, which indicates formation of four pairs of disulfide bridges. Then, the pH of folding buffer was adjusted to 2 with TFA and purified by semi-preparative RP-HPLC (yield = 15-25%). The purity of the folded HsTX1 analogies were confirmed by

analytical RP-HPLC and ESI-MS.

All Fmoc-amino acids and reagents were purchased commercially and used without further purification. Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Trp(5-F)-OH, Fmoc-Trp(7-F)-OH , Fmoc-Phe(4-F)-OH, 5-Chloro-1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HCTU), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b] pyridinium 3-oxide hexafluorophosphate (HATU), Rink-amide resin (loading = 0.37 mmol/g) was purchased from Tianjin Nankai Hecheng Technology Co., Ltd. N, N-diisopropylethylamine (DIEA) were purchased from GL Biochem. N, N-dimethylformamide (DMF), dichloromethane (DCM), and high-performance liquid chromatography (HPLC)-grade acetonitrile were purchased from Chron Chemicals Co., Ltd. HPLC-grade trifluoroacetic acid (TFA, 99.5%) was purchased from J&K Scientific Ltd. Piperidine, phenol and diethyl ether were purchased from the Sinopharm Chemical Reagent Co., Ltd. Trifluoroacetic acid, 1,2-ethanedithiol, thioanisole and phenylsilane were purchased from Energy Chemical.

Analytical reversed-phase high-pressure liquid chromatography（RP-HPLC）was performed using Shimadzu (Prominence LC-20AT) instrument equipped with a dual wavelength (214 nm, 254 nm) UV-VIS detector SPD-20A and an analytical column (Reprosil-Pur Basic-C18, 4.6 ×250 mm, 5 μm particle size, flow rate 1.0 ml/min). Semi-preparative RP-HPLC was performed using Shimadzu Prominence HPLC LC-20AT equipped with a dual wavelength (214 nm, 254 nm) UV-VIS detector SPD-20A and a Semi-preparative column (Reprosil-Pur Basic-C18, 10 ×250 mm, 5 μm particle size, flow rate 3.5 ml/min). Buffer A was 0.1% TFA in acetonitrile, and buffer B was

0.1% TFA in water. Both Buffers were sonicated for 15 min before use. Synthetic peptides were characterized by electrospray ionization mass spectrometry (ESI-MS, Shimadzu LCMS-2020).

1.2 Cloning, Protein Expression, and Purification of Kv1.3.

The gene for human Kv1.3(UniProtKB accession no. P22001), lacking the first 52 residues, and human Kvβ2.1(UniProtKB accession no. Q13303) were cloned for coexpression into a pFastBac dual vector (Invitrogen) with a FLAG tag (DYKDDDDK) at the amino terminus of Kv1.3. The expression construct expressing Kv1.3–Kvβ2.1 was transformed into DH10Bac cells to produce bacmid. For large-scale expression, baculovirus after two rounds of amplification was used to infect Sf9 cells at 2 x 10^6cells/ml cultured in SIM SF medium (Sino Biological Inc.) at 27 °C. Infected Sf9 cells were cultured for 60 h before harvesting. The cell pellet from 1 L of culture was resuspended in lysis buffer (20 mM HEPES, pH 7.5, 150 mM KCl, and 10% glycerol). The suspension was supplemented with 1.5% (wt/vol) n-dodecyl-β- D -maltopyranoside (DDM;Anatrace), 0.3% (wt/vol) cholesteryl hemisuccinate (Sigma), and protease inhibitor cocktail. After extraction at 4°C for 2 h, the insoluble fraction was removed by ultracentrifugation at 180,000 × g for 45 min at 4°C, and the supernatant was incubated with anti-FLAG G1 affinity gel (GenScript) for 1 h at 4°C. The resin was then collected and washed with wash (W) buffer (20 mM HEPES, pH 7.5, 150 mM KCl, and0.1% GDN). The proteins were eluted with W buffer composed of 200 μg/mL FLAG peptide. After elution, the proteins were concentrated and further purified on a Superose 6 increase column (Cytiva) equilibrated with 20 mM HEPES, pH 7.5, 150 mM KCl, and 0.005%GDN. Peak fractions of the protein complex were collected, analyzed by SDS-PAGE , flash-frozen in liquid nitrogen, and stored at -80°C.

1.3 Electrophysiology

DNA encoding hKV1.3 was cloned into a pcDNA3.1/Zeo(+) vector. Chinese hamster ovary (CHO) cells were cultured in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, GIBCO), 1% Penicillin-Streptomycin-Glutamine (GIBCO) at 37°C in a 5% CO2 incubator. Plasmids carrying K V 1.3 channels and plasmid carrying EGFP were transiently transfected into CHO cells using Lipofectamine 3000(Invitrogen). After incubation for 5 h, the cells were transferred to poly-L-lysine-coated glass coverslips for culture for another 24-48 h in fresh medium. They were then used for electrophysiological recording.

For whole-cell patch clamp recordings, The electrodes were pulled from thick-walled borosilicate glass capillaries with filaments (World Precision Instruments，Inc.) on a four-stage puller (P-1000, Sutter Instruments) and had resistances of 3-5 MΩ. Data were obtained using EPC10 patch clamp amplifier (HEKA Electronics) in whole-cell configuration under voltage-clamp mode (current measurement) at room temperature Kv1.3 currents were elicited by repeated 200-ms pulses from holding potential (-80 mV) to 40 mV, applied at intervals of 30s to avoid cumulative inactivation. After three stable records of currents, HsTX1 was applied to the patched cell through a Y-tube perfusion system and the degree of block determined. Recording solution compositions are as follows: the external solution: 150 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES (pH 7.4, ~308 mOsm), and the internal solution: 140 mM KCl, 10 mM NaCl, 5 mM EGTA, and 10 mM HEPES (pH 7.4, ~297 mOsm). All chemicals were obtained from Sigma.

1.4 Conformational analysis by CD

The CD spectra of peptides was measured by JASCO J-1700 CD spectroscopy ((JASCO, Japan) at

room temperature. The concentration of all peptides was 100 μg/mL. Detection wavelength range was 190– 240 nm; bandwidth was 1 nm; and scanning speed was 200 nm/min. Results are expressed as molar ellipticity ($[\theta]_M$) per residue.

1.5 ^{19}F NMR spectroscopy

^{19}F-NMR screening experiments were performed using an Avance III (600 MHz) Bruker spectrometer equipped with a TCI ^{1}H/^{13}C/^{15}N cryogenic probe, with the 1H channel tuned to the ^{19}F Larmor frequency. The experiments were performed at 298 K in 500 μL samples in 5 mm NMR tubes. The samples were dissolved in buffer consisting of 20 mM HEPES and 150 mM KCl with 10% D_2O and 0.005% (w/vol) GDN (Anatrace). The experiment was recorded at a temperature of 298 K, with an acquisition time of 180 ms and a recycle delay of 1 s.^{19}F chemical shifts were referenced to an internal compound of trifluoro-acetic acid (TFA, 75.39 ppm). ^{19}F relaxation-filtered one-dimensional NMR was performed using a T_2 filter-based Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with transverse relaxation durations 10 ms, which consists of a hard 90° excitation pulse followed by a series of 180° refocusing pulses that create an echo. The 1D ^{19}F-NMR spectra were processed with MestReNova (Mestrelab Research S.L.) and Topspin 4.3.0 software (Bruker).

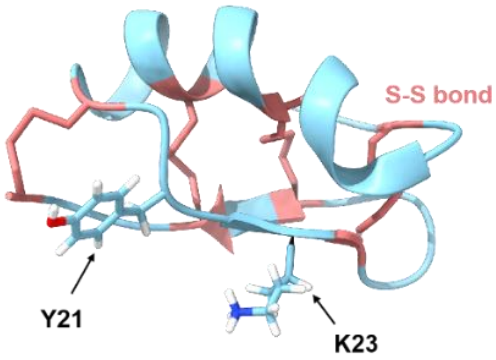

Figure S1. Solution NMR structure of scorpion toxin HSTX1 (PDB 1QUZ). The disulfide bridges were shown in red. The residue Y21 and K23 were shown as sticks.

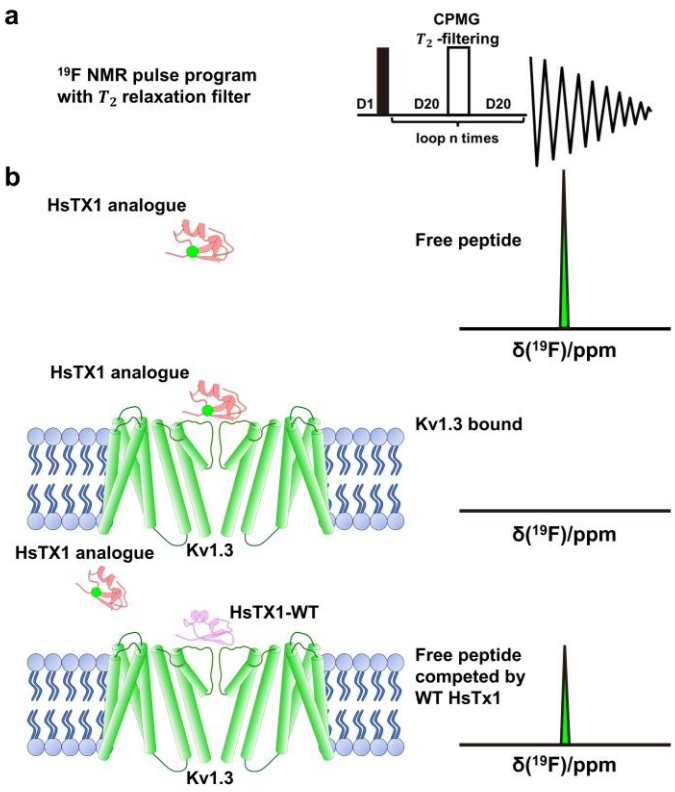

Figure S2. (a) ^{19}F NMR pulse schemes for the T_2 relaxation-filtered NMR experiments. The black bar indicates the 90° pulse, and the white bar represents the 180° pulse. (b) A sharp ^{19}F NMR signal was observed for the free peptide in the buffer. With the addition of Kv1.3, the ^{19}F NMR signal of the peptide was attenuated and even disappeared due to binding to the protein. The introduction of a competing ligand resulted in partial recovery of the ^{19}F signal of the peptide, which was attributed to dissociation of the peptide from Kv1.3.

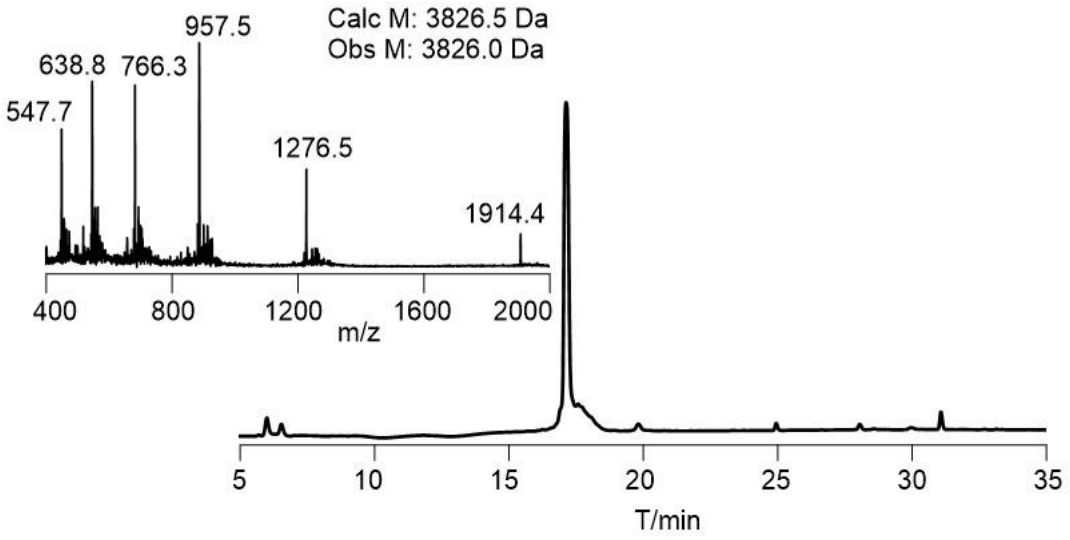

Figure S3. Analytical HPLC chromatogram of the linear full-length precursor of HsTX1 WT. The main peak in the HPLC profile corresponds to linear full-length precursor of HsTX1 WT. The gradient used for analytical HPLC is 1-61% buffer B in buffer A in 30 min. The ESI-MS spectrum gave an observed mass of 3826.0 Da (calcd 3826.5 Da, average isotopes).

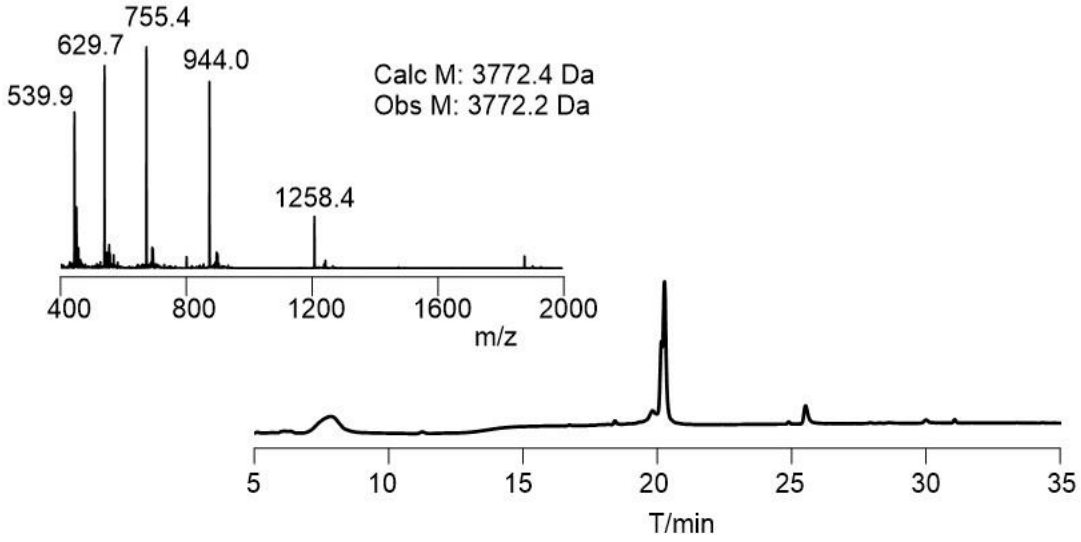

Figure S4. Analytical HPLC chromatogram of the linear full-length precursor of peptide **1**. The main peak in the HPLC profile corresponds to linear full-length precursor of peptide **1**. The gradient used for analytical HPLC is 1-61% buffer B in buffer A in 30 min. The ESI-MS spectrum gave an observed mass of 3772.4 Da (calcd 3772.2 Da, average isotopes).

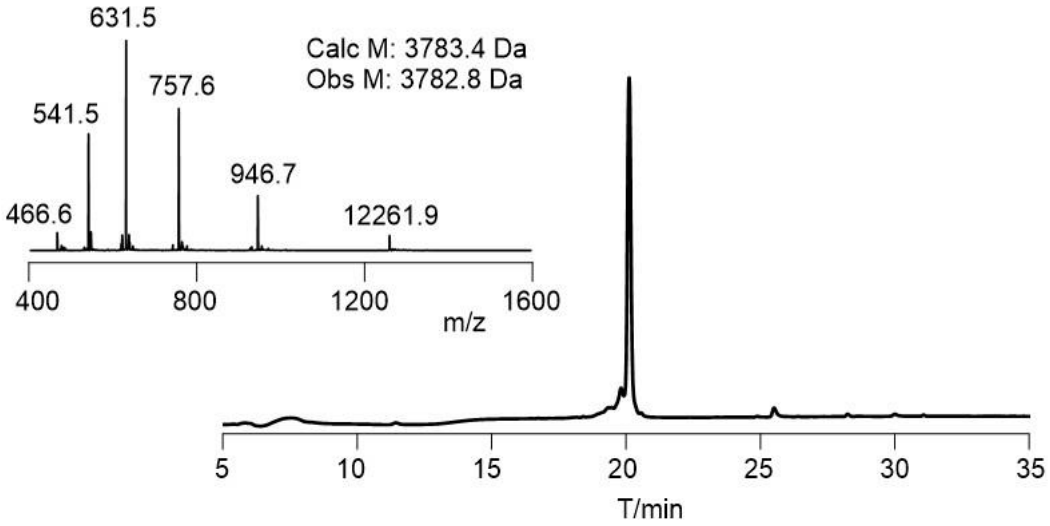

Figure S5. Analytical HPLC chromatogram of the linear full-length precursor of peptide **2**. The main peak in the HPLC profile corresponds to linear full-length precursor of peptide **2**. The gradient used for analytical HPLC is 1–61% buffer B in buffer A in 30 min. The ESI-MS spectrum gave an observed mass of 3782.8 Da (calcd 3783.4 Da, average isotopes).

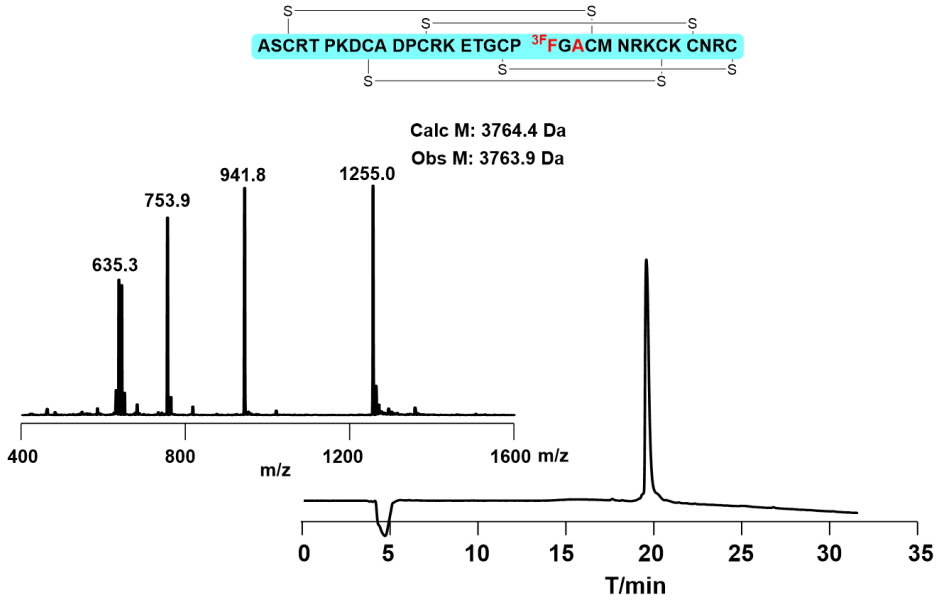

Figure S6. Analytical HPLC chromatogram of peptide **4**. The main peak in the HPLC profile corresponds to correctly folded peptide **4**. The gradient used for analytical HPLC is 1-61% buffer B in buffer A in 30 min. The ESI-MS spectrum gave an observed mass of 3764.4 Da (calcd 3763.9 Da, average isotopes).

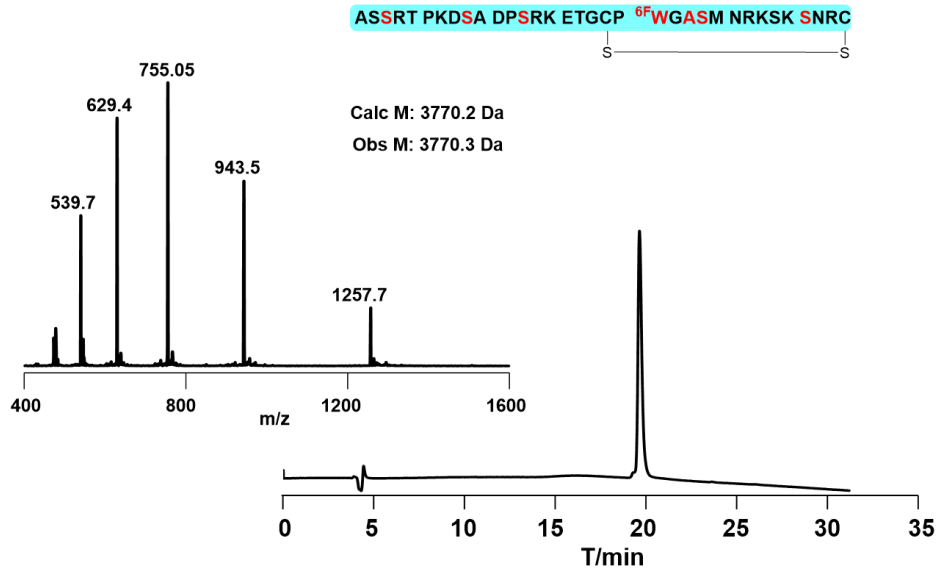

Figure S7. Analytical HPLC chromatogram of peptide **5**. The main peak in the HPLC profile corresponds to correctly folded peptide **5**. The gradient used for analytical HPLC is 1-61% buffer B in buffer A in 30 min. The ESI-MS spectrum gave an observed mass of 3770.2 Da (calcd 3770.3 Da, average isotopes).

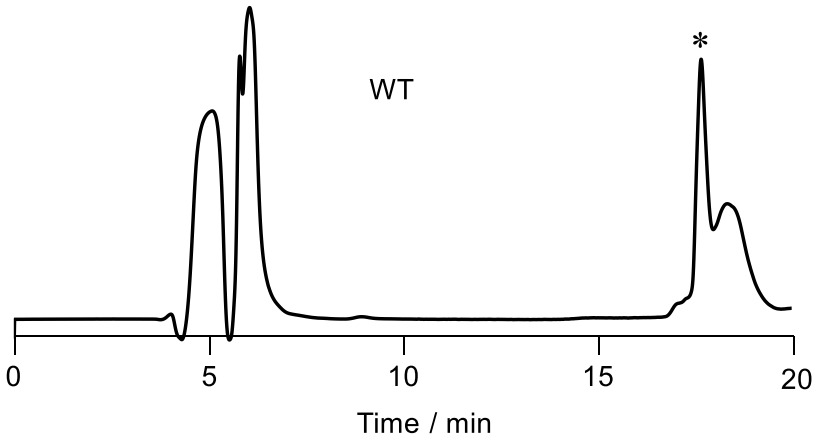

Figure S8. HPLC analysis traces for folding of HsTX1 WT. The main peak in the HPLC profile corresponds to correctly folded HsTX1 WT. The gradient used for analytical HPLC is 1-61% buffer B in buffer A in 30 min. Peak corresponds to the expected product was marked with a star.

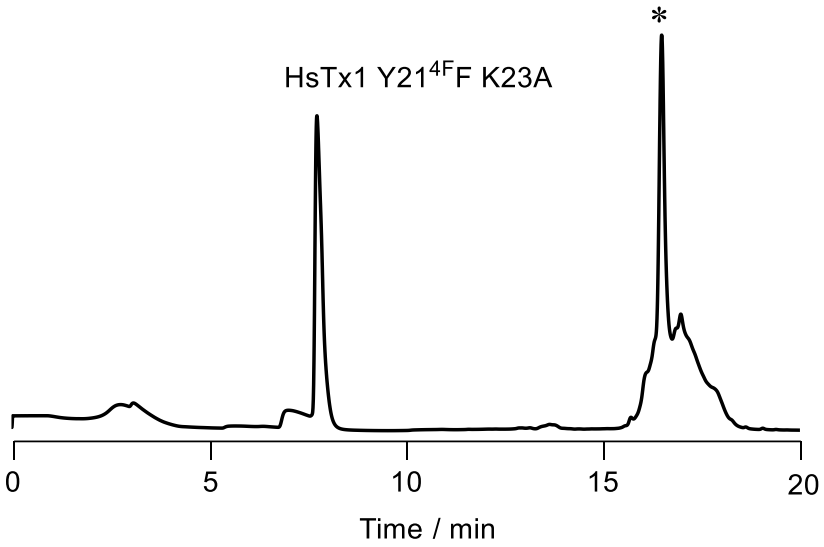

Figure S9. HPLC analysis traces for folding of peptide **1**. The main peak in the HPLC profile corresponds to correctly folded peptide **1**. The gradient used for analytical HPLC is 1-61% buffer B in buffer A in 30 min. Peak corresponds to the expected product was marked with a star.

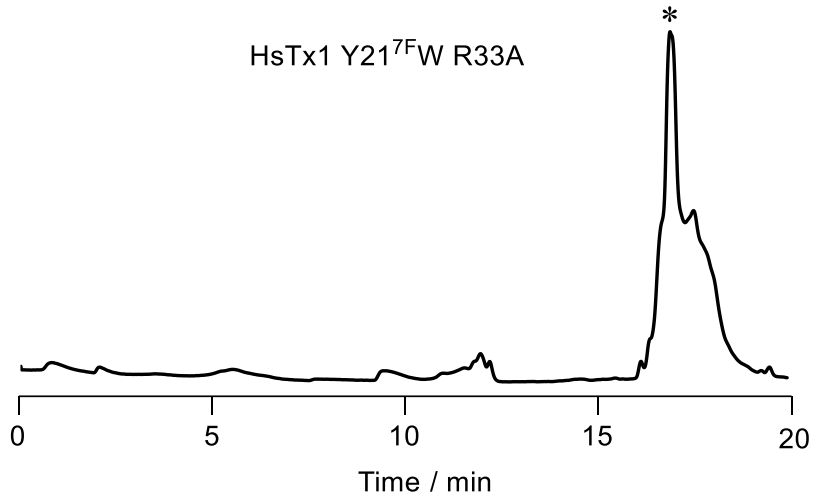

Figure S10. HPLC analysis traces for folding of peptide **2**. The main peak in the HPLC profile corresponds to correctly folded peptide **2**. The gradient used for analytical HPLC is 1-61% buffer B in buffer A in 30 min. The main peak of HPLC profile corresponds to the expected product was marked with a star.

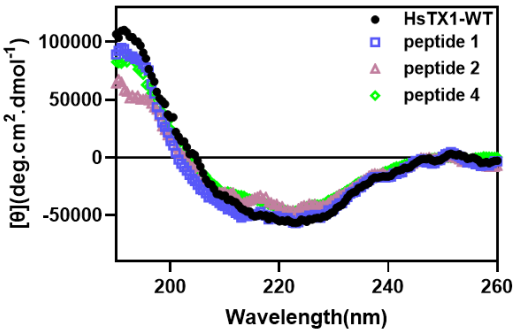

Figure S11. CD spectrum of peptide **1**, **2**, **4** and HsTX1-WT. The concentration of all peptides was 100 μg/ml in 20 mM sodium phosphate buffer, pH 7.1. Spectra were recorded with a 1-mm-pathlength cell.

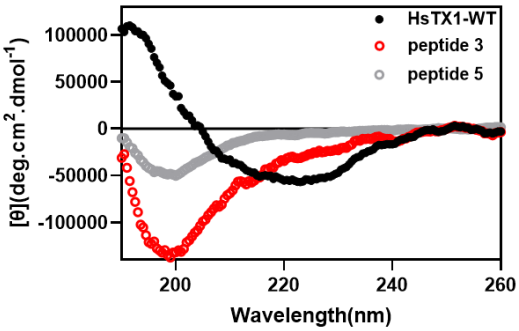

Figure S12. CD spectrum of peptide **3**, **5** and HsTX1-WT. The concentration of all peptides was 100 μg/ml in 20 mM sodium phosphate buffer, pH 7.1. Spectra were recorded with a 1-mm-pathlength cell. Unlike four disulfide bridges HsTX1-WT, peptides **5** has only one pair of disulfide bridges (C19/34). The CD spectra of peptide **5** was similar with linear peptide **3,** but different from four disulfide bridge HsTX1-WT, suggesting that disulfide bonds play a critical stabilizing role in HsTX1 structure.

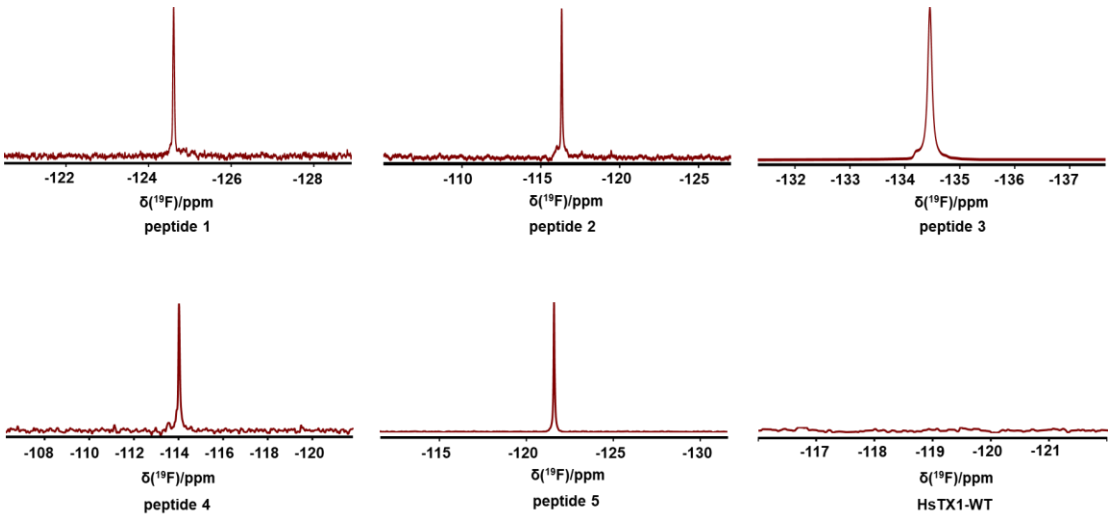

Figure S13. ^{19}F chemical shifts of the individual peptides. In the detergent solution, all peptides exhibit sharp signals in the ^{19}F-NMR spectrum.

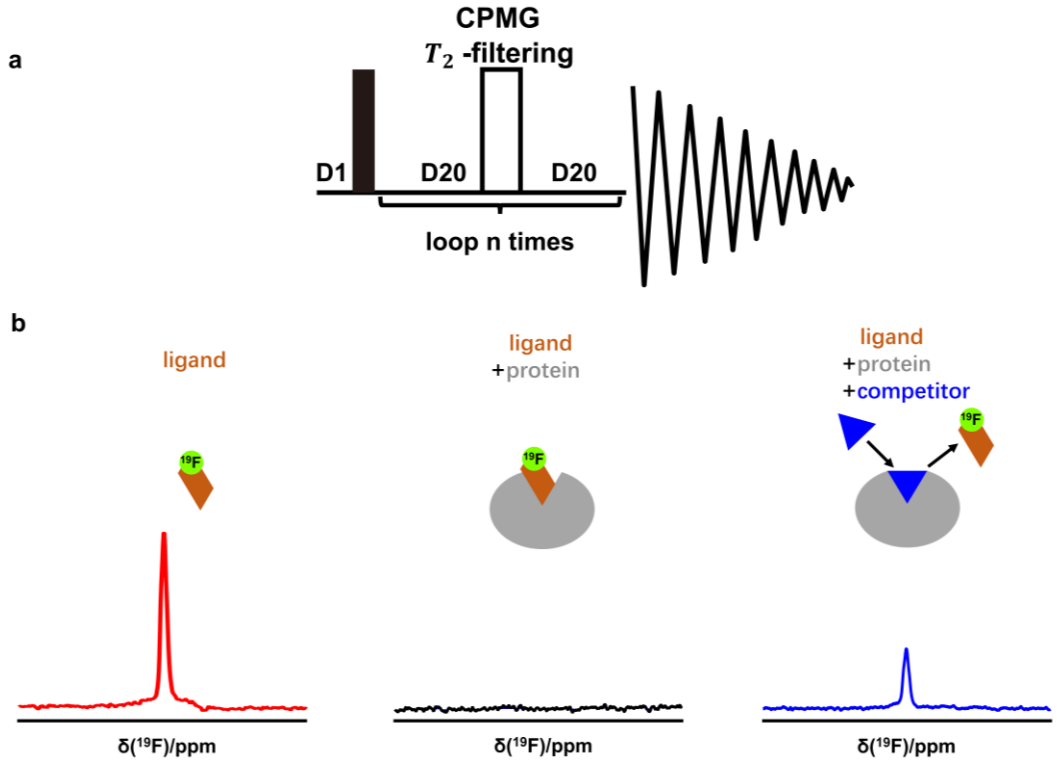

Figure S14. (a) ^{19}F NMR pulse Scheme for ^{19}F T_2-filter experiments. The black and white bars indicate 90-degree and 180-degree pulses, respectively. (b) 1D ^{19}F NMR spectrum of peptide in buffer (left), the target protein bound (middle), the competition compound addition (right). Protein ligand binding was observed by the attenuated resonance intensity of ^{19}F NMR from ^{19}F incorporated ligand.

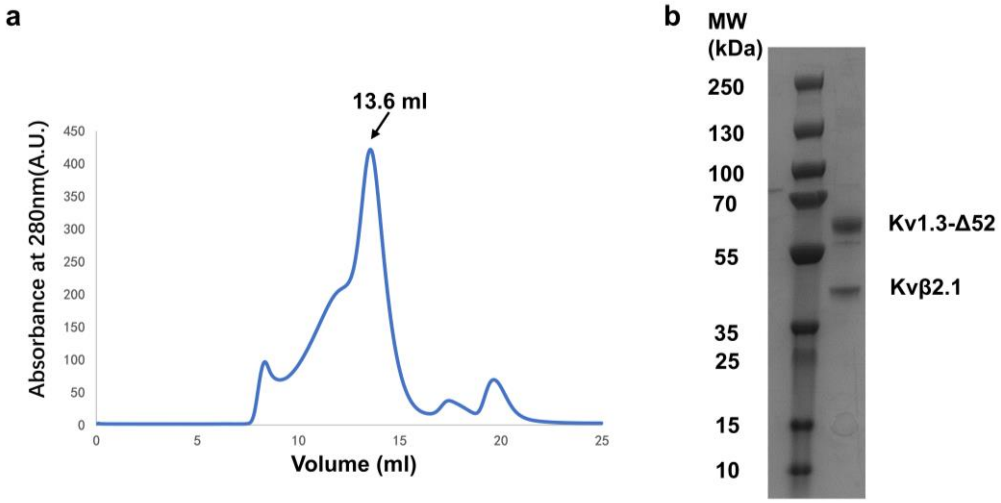

Figure S15. Expression and purification of human Kv1.3. (a)Size exclusion chromatogram of the complex. (b) SDS-PAGE of main fractions collected from size exclusion chromatography.

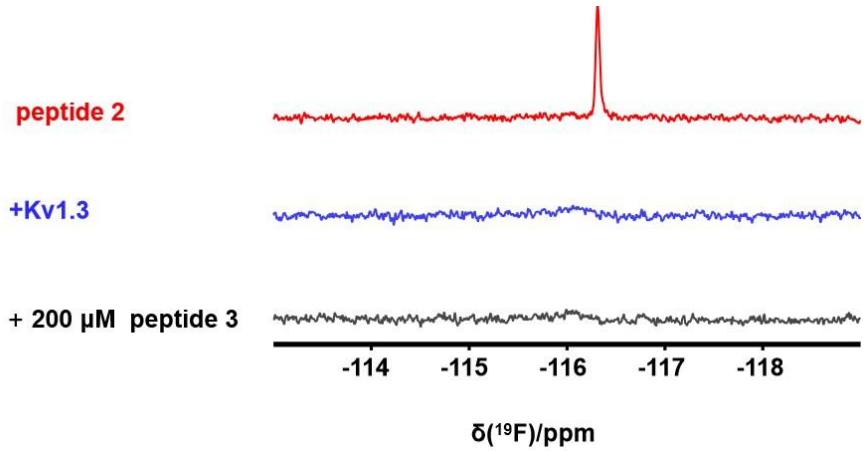

Figure S16. The competitive binding assay using a non-competitive inhibitor. The ^{19}F NMR spectra of peptide **2** (8 μM) in the free state, with Kv 1.3 (8 μM) addition and with a non-competitive inhibitor. The ^{19}F-NMR signal fail to restore when 8 times (200 μM) peptide **3** was added as a competitive inhibitor. The result demonstrated that the competitive binding assay was specific and affinity-dependent, and further confirming the reliability of ^{19}F-NMR-based peptide ligand screening.

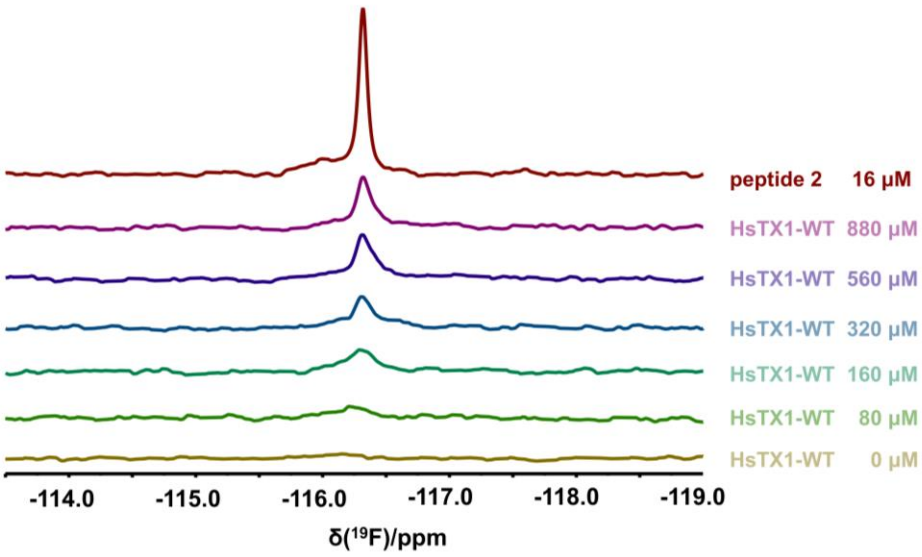

Figure S17. In the NMR titration experiments, the ^{19}F-NMR signal of peptide **2** (16 μM) disappeared upon the addition of 20 μM Kv1.3. However, its resonance intensity gradually increased with the stepwise addition of HsTX1-WT, suggesting the competition of peptide **2** from Kv1.3 by HsTX1-WT.

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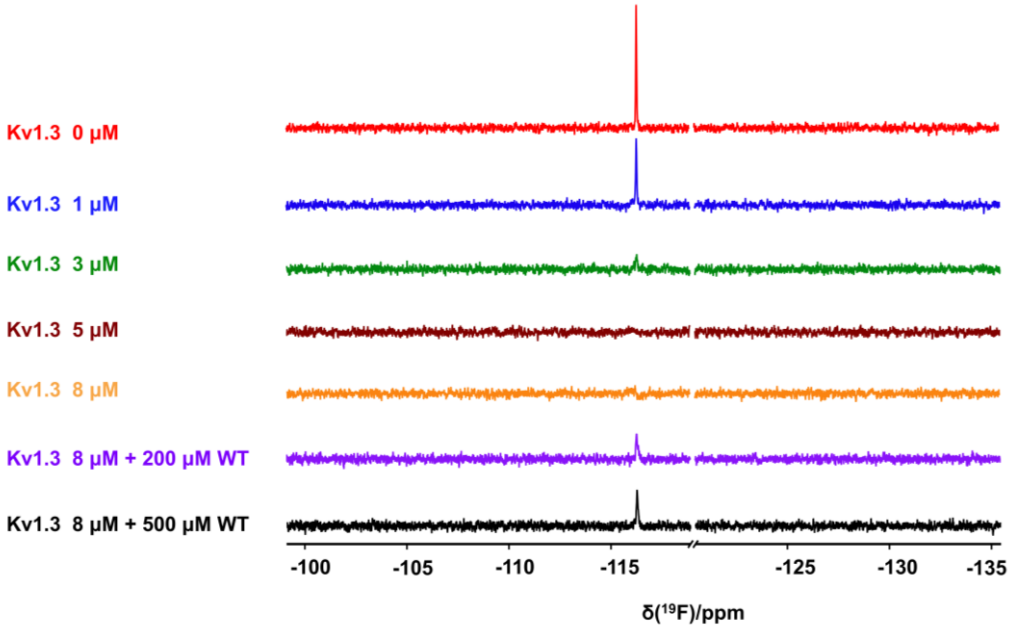

Figure S18. The NMR titration experiments of peptide **2** (8 μM) with increasing concentration of purified Kv1.3 protein. With the addition of Kv1.3 protein, the intensity of the ^{19}F-NMR signals of peptide **2** was gradually decreased. The intensity of peptide **2** could be partially restored upon the addition of wild-type HsTX1 competitor. When 8 μM Kv1.3 protein was added, the ^{19}F-NMR signals of peptide 2 was completely disappeared.

a

Kv1.3 (μM)	$(Y_{max}-Y)/Y_{max}$
0	0
1	0.292202
3	0.759658
5	1
8	1

b

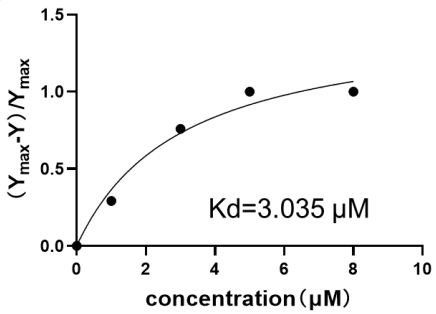

Figure S19. The binding capability of ^{19}F-labeled HsTx1 peptide with purified Kv1.3 protein was measured with the NMR titration experiments. (a) The ratios of the peak integration of the ^{19}F-NMR signals of peptide **2** in Figure S16 are shown. "Ymax" is the ^{19}F NMR spectra of peak integration **2** in the free state, "Y" is the ^{19}F NMR spectra peak integration of peptide 2 with the addition of Kv1.3 protein. (b) Using the software GraphPad for curve fitting, the resulting Kd Value was determined to be 3.05 μM.

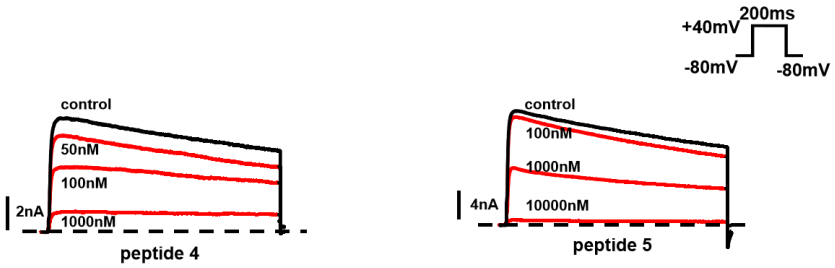

Figure S20. Analysis of the inhibitory effects of HsTX1 analogues on Kv1.3 function via patch-clamp electrophysiology experiments. Superimposed current traces of Kv1.3 channels recorded in whole-cell patch clamp experiments with different concentrations of peptide **4** and peptide **5**. The cells were held at −80 mV and stepped to +40 mV for 200 ms with an interpulse interval of 30 s.

Peptides	IC_{50}
HsTX1-WT	59.31±12.30 pM
peptide **1**	0.50±0.047 nM
peptide **2**	108.30±16.21 nM
peptide **3**	N.D
peptide **4**	111.50±25.44 nM
peptide **5**	924.90±253.8 nM

Table S1. IC_{50} values of peptides on Kv1.3. IC_{50} values of peptides on Kv1.3 measured using whole-cell patch-clamp recording. All peptides were tested three to five times at five concentrations and IC_{50} values were shown as mean values ± SEMs.