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Pharmacological activity and NMR solution structure of the leech peptide HSTX-I

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Abstract

The role of voltage-gated sodium (Na_v) channels in pain perception is indisputable. Of particular interest as targets for the development of pain therapeutics are the tetrodotoxin-resistant isoforms Na_v1.8 and Na_v1.9, based on animal as well as human genetic studies linking these ion channel subtypes to the pathogenesis of pain. However, only a limited number of inhibitors selectively targeting these channels have been reported. HSTX-I is a peptide toxin identified from saliva of the leech *Haemadipsa sylvestris*. The native 23-residue peptide, stabilised by two disulfide bonds, has been reported to inhibit rat Na_v1.8 and mouse Na_v1.9 with low micromolar activity, and may therefore represent a scaffold for development of novel modulators with activity at human

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

tetrodotoxin-resistant Nav isoforms. We synthetically produced this hydrophobic peptide in high yield using a one-pot oxidation and single step purification and determined the three-dimensional solution structure of HSTX-I using NMR solution spectroscopy. However, in our hands, the synthetic HSTX-I displayed only very modest activity at human Nav1.8 and Nav1.9, and lacked analgesic efficacy in a murine model of inflammatory pain.

Keywords

Voltage-gated sodium channels; Electrophysiology; NMR; Leech; Chronic pain

1. Introduction

Chronic pain is a debilitating neurological disease that affects 20% of the world's population and is associated with a large financial burden. With the alarming rise in fatalities related to opioid-overuse there is an urgent need for novel, non-addictive pain therapeutics. Voltage-gated sodium (Nav) channels, pore-forming proteins found on the surface of excitable cells that are integral to the initiation and propagation of action potentials [1], represent one promising class of drug targets for pain treatment [2–4]. Although a number of Nav channel modulators have successfully been used in the clinical as analgesics, for example lidocaine, the non-selective nature and resultant side-effects of these modulators has limited their therapeutic utility.

Nine different pore-forming α -subunits are encoded by the *SCNxA* genes ($x = 1-5, 8-11$) and give rise to Nav channel subtypes Nav1.1–1.9. These subtypes can be pharmacologically distinguished based on their sensitivity to tetrodotoxin (TTX), a toxin isolated from the puffer fish, with Nav1.1–1.4 and Nav1.6–1.7 classified as TTX-sensitive (TTX-s), and Nav1.5 and Nav1.8–1.9 characterised as TTX-resistant (TTX-r) channels [5]. Several of these subtypes – including Nav1.1, 1.3, 1.6, 1.7, 1.8 and 1.9 – are expressed on sensory neurons, where they are involved in generation and propagation of action potentials. Nav1.8 and Nav1.9 in particular have long been considered attractive analgesic targets owing to their preferential expression on small-diameter nociceptors as well as their unique biophysical properties [6,7]. Specifically, Nav1.8 is characterised by rapid repriming kinetics and relatively depolarised activation and inactivation thresholds, making this subtype ideally suited to maintaining repetitive firing in response to sustained depolarisations [8]. A range of inflammatory mediators have been shown to increase expression and/or function of Nav1.8, and the major role of this channel in various pain states has been confirmed by gene deletion or knockout studies [9,10]. In contrast, Nav1.9 is characterised by very hyperpolarised activation thresholds, slow activation and ultraslow inactivation kinetics [11]. These properties, including a large overlap in the voltage-dependence of activation and fast inactivation, position Nav1.9 as an amplification channel that likely prolongs small depolarisations close to resting membrane potential, and that may contribute to setting the resting membrane potential. Indeed, gain-of-function mutations of Nav1.9 can lead to either painful or painless conditions, depending on the degree to which the resultant window currents depolarise the resting membrane potential of sensory neurons [12]. Accordingly, Nav1.8 and Nav1.9 have been identified as key players

in nociceptive signalling and are considered to be important targets for the development of novel analgesics.

Toxins from venomous creatures have provided a rich source of Na_v modulators that often afford high potency and efficacy for their targets as well as intrinsic stability through complex disulfide networks. However, compared to other Na_v subtypes such as Na_v1.7, relatively few selective Na_v1.8 inhibitors [13,14], and even fewer molecules with activity at Na_v1.9 [15], have been reported.

Recently, native HSTX-I, a small peptide toxin isolated from the leech *Haemadipsa sylvestris*, was shown to selectively modulate TTX-r channels, specifically Na_v1.8 and Na_v1.9, expressed in sensory neurons [16]. In the present study, we thus sought to explore the pharmacological activity and analgesic potential of synthetic HSTX-I. We developed a one-pot oxidation and single purification peptide synthesis protocol, determined the 3D NMR structure and evaluated the pharmacological and analgesic activity of this novel leech-derived peptide toxin.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated all chemicals were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MI, USA). Amino acids were purchased from Chem-Impex International (Chem-Impex International, Inc., Wood Dale, IL, USA).

2.2. Peptide synthesis of HSTX-I

HSTX-I (ACKEYWECGAFLFCIEGICVPMI*) peptides were synthesised by solid phase peptide synthesis (SPPS) on a Symphony automated peptide synthesizer (Gyros Protein Technologies Inc., Tucson, AZ, USA), on rink-amide resin (Merck, Bayswater, VIC, Australia) at 0.125 mmol scale to produce a C-terminal amidated peptide (*) using 9-fluorenylmethoxycarbonyl (Fmoc) methodology. Side chain protecting groups used were Cys (Acm/Trt), Lys (Boc), Tyr (tBu), Trp (Boc) and Glu (OtBu). The peptide was cleaved from resin and side chains simultaneously removed following a 2 h incubation with 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane and 2.5% water (H₂O). Excess TFA was evaporated under vacuum and reaction precipitated with ice-cold diethyl ether. Precipitant was collected on 20 µm frit (Phenomenix, Torrance, CA, USA) via gravity filtration and dried for 1 h to remove excess ether. Crude peptide was dissolved in 50% acetonitrile (ACN) and 0.05% TFA and lyophilised.

2.3. Oxidation

Protecting groups trityl (Trt) and acetamidomethyl (Acm) were employed for regioselective protection of three HSTX-I peptides with different connectivities (C2/C14 and C8/C19, C2/C8 and C14/C19, C2/C19 and C8/C14). The peptides were dissolved to 0.1 mg/mL in 50% ACN and the first cysteine pair was oxidised using air oxidation while stirring for 10 min. Subsequently, 40 nM iodine was added to remove the Acm protecting groups, and the second cysteine pair was oxidised after 5 min incubation with stirring. Reactions

were quenched with ascorbic acid and solution diluted to 25% ACN for reversed-phase high-performance liquid chromatography (RP-HPLC) purification. Oxidised peptides were purified by RP-HPLC on a Phenomenex C18 column over a linear gradient between 25 and 75% Solvent B (90% ACN; 0.05% TFA) over 45 min at 8 mL/min. Fractions containing the correct molecular mass confirmed by mass spectrometry (MS) were pooled, lyophilised and stored at -20°C until used.

2.4. Nuclear magnetic resonance (NMR)

All NMR experiments were conducted at 298 K and pH 4.0 unless otherwise stated. HSTX-I (2 mg/mL) was dissolved in 33% deuterated ACN (ACN-d_3 ; Cambridge Isotope Laboratories, Cambridge, UK)/67% H_2O (v/v). One-dimensional (1D) ^1H spectra and two-dimensional (2D) total correlated spectroscopy (TOCSY) (80 ms mixing time) and nuclear Overhauser effect spectroscopy (NOESY) (200 ms mixing time) experiments were run and data collected on a Bruker Avance 600 MHz NMR spectrometer equipped with a cryoprobe (Bruker, Sydney, NSW, Australia). Additional NMR data collected for solution structure calculations included ^1H - ^{15}N HSQC in 33% ACN-d_3 /67% H_2O (v/v) as well as 2D TOCSY at various temperatures (283–303 K) and ^1H - ^{13}C HSQC in 33% ACN-d_3 /67% D_2O (v/v). Slow exchanging amide protons were identified from a series of 1D and TOCSY spectra collected following the dissolution in D_2O . Although D_2O exchange was monitored over 24 h, slow exchangers were identified as amide protons remaining beyond 4 h.

2.5. NMR analysis

NMR spectra were processed using TopSpin 3.5 (Bruker, Sydney, NSW, Australia). Peptide sequences were sequentially assigned, and a list of inter-proton distances were generated using CCPNMR Analysis 2.1.5.67. Secondary structure was predicted by comparing calculated H_α chemical shifts to reported average random coil H_α shifts. H_α , C_α , C_β , HN chemical shifts derived from TOCSY, NOESY, ^1H - ^{13}C HSQC and ^1H - ^{15}N HSQC spectra were used to calculate backbone (Φ and ϕ) dihedral angle constraints in TALOS-N [17]. Peak assignments were refined in CYANA 3.97 following several rounds of ANNEAL calculations. D_2O exchange and temperature coefficient experiments, in combination with NOE intensities were included to determine H-bond restraints and χ_1 side chain dihedral angle restraints were derived from DISH. Protocols in the RECOORD database were used in CNS to calculate 100 structures, which were further refined in a water shell [18,19]. A final set of 20 structures were chosen based on the lowest energy, fewest violations, and the best MolProbity scores [20].

2.6. Whole-cell patch-clamp electrophysiology

Automated whole-cell patch-clamp recordings were performed with a QPatch-16 automated electrophysiology platform (Sophion Bioscience, Ballerup, Denmark) on HEK293 cells heterologously expressing human $\text{Nav}_1.7$ (SB Drug Discovery, Glasgow, UK) and CHO cells heterologously expressing human $\text{Nav}_1.8$ (ChanTest, Cleveland, OH, USA). Cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and the selection antibiotics as recommended by the manufacturer. Cells were grown in an incubator at 37°C with 5% CO_2 and passaged every 3–4 days (at 70–80% confluency) using TrypLE Express (Thermo Fisher Scientific,

Scoresby, VIC, Australia). Prior to recordings, cells were dissociated with TrypLE Express and resuspended in Ham's F-12 medium (Gibco - Thermo Fisher Scientific) supplemented with 25 mM HEPES, 100 U/mL Penicillin-Streptomycin (Gibco - Thermo Fisher Scientific) and 40 µg/mL trypsin inhibitor from *Glycine* max (soybean), and allowed to recover with stirring for 30 min.

The extracellular solution contained (in mM) 145 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, pH to 7.4 with NaOH (305 mOsm/L). The intracellular solution contained (in mM) 140 CsF, 1 EGTA, 5 CsOH, 10 HEPES, and 10 NaCl, pH to 7.3 with CsOH (320 mOsm/L). HSTX-I was diluted in extracellular solution with 0.1% BSA to 90 µM and incubated for 5 min. Na_v1.7 currents were elicited by a 50 ms pulse to -20 mV from a holding potential of -90 mV. Na_v1.8 currents were elicited by a 50 ms pulse to +10 mV from a holding potential of -90 mV. Recordings were taken at ambient room temperature (22 °C) after 5 min incubations. Peak current post HSTX-I addition (I) was normalised to buffer control (I₀).

2.7. Isolation of SCG neurons

Human Na_v1.9 channel construct with a "stopGo" 33 amino acid 2A linker previously described [21] permits GFP and the Na_v1.9 channel to be produced as independent proteins from the same mRNA transcript. Superior cervical ganglion (SCG) neurons which lack endogenous TTX-r currents were harvested from 0 to 5 day-old Sprague Dawley rats as previously described [22], and transfected by electroporation with a wild-type human Na_v1.9 construct. Culture medium was supplemented with 50 ng/mL mouse nerve growth factor (Alomone Laboratories, Jerusalem, Israel) and 50 ng/mL recombinant human glial cell line-derived neurotrophic factor (PeproTech, Rocky Hill, NJ, USA), and the SCG neurons were maintained at 37 °C in a 5% CO₂ incubator for 12–18 h then transferred to 30 °C for 30 h before recording. All animal procedures to isolate SCG neurons were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the IACUC of the Veterans Administration Connecticut Healthcare System.

2.8. Electrophysiological recordings of SCG neurons

Voltage-clamp recordings were performed on isolated SCG neurons using an EPC-10 double amplifier and Patchmaster software (HEKA Elektronik, Germany). Recordings were obtained from SCG neurons at 22 ± 1 °C, 40–50 h after plating. Fire-polished electrodes were fabricated from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a Sutter Instruments P-97 puller and had a resistance of 1.1–1.5 MΩ. Pipette potential was adjusted to zero before seal formation. Liquid junction potential was not corrected. Series resistance compensation (80–85%) was applied to reduce voltage errors. Currents were acquired at least 5 min after establishing whole-cell configuration. Recordings were acquired at 2.9 kHz with a low-pass Bessel filter, and digitized at 50 kHz. Linear leak currents were subtracted with P/6 subtraction.

For recording human Na_v1.9 currents from SCG neurons the following extracellular bath solutions were used (in mM): 140 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 5 CsCl, 20 TEA-Cl, 0.1 CdCl₂, 1 4-aminopyridine, pH to 7.3 with NaOH (322 mOsm/L). Pipette

solution contained the following (in mM): 135 CsF, 5 NaCl, 10 CsCl, 5 EGTA, and 10 HEPES, 3 Mg-ATP, pH to 7.3 with KOH (314 mOsm/L). To isolate hNa_v1.9 currents in voltage-clamp recordings, cells were held at -100 mV and stepped to -45 mV for 50 ms following a prepulse of 1000 ms at -120 mV. TTX (1 μ M) was included in the bath solution to block endogenous TTX-s sodium channels.

HSTX-I was dissolved in 100% DMSO as a 10 mM stock. Control solution consisted of 0.1% DMSO. Cells with stable currents (< 10% change) during baseline recordings were perfused with 10 μ M HSTX-I for 3 min (> 3 times bath volume), then static bath for an additional 3 min. This was followed by a washout period (5 min). Extracellular bath solution was superfused at a flow rate of 1 mL/min using a pressure regulated perfusion system (AutoMate Scientific, Berkley, CA, USA), and delivered through a 250 μ m perfusion pencil. Solutions containing control or HSTX-I were delivered through the perfusion system.

2.9. Expression of voltage-gated ion channels in *Xenopus laevis* oocytes

For the expression of human, Na_v channels (Na_v1.5, Na_v1.7, Na_v1.8, Na_v1.9_C4 and the auxiliary subunit β 1) in *Xenopus* oocytes, the linearized plasmids were transcribed by using the T7 or SP6 mMessage-mMachine Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The construction of Na_v1.9_C4 has been previously described [23]. *Xenopus laevis* frogs were purchased from CRB (Centre de Ressources Biologiques Xénopes, France). The harvesting of stage V–VI oocytes from anesthetized female *X. laevis* frogs was previously described [24]. Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng/nL by using a microinjector (Drummond Scientific Company, Broomall, PA, USA). The oocytes were incubated in a solution containing (in mM) the following: 96 NaCl, 2 KCl, 1.8 CaCl₂, 2 MgCl₂ and 5 HEPES (pH 7.4). This solution was supplemented with 50 mg/L gentamicin sulfate. The use of the frogs was in accordance with the license number LA1210239 of the Laboratory of Toxicology & Pharmacology, University of Leuven. All animal care and experimental procedures agreed with the guidelines of 'European convention for the protection of vertebrate animals used for experimental and other scientific purposes' (Strasbourg, 18.III.1986).

2.10. Electrophysiological recordings in *Xenopus laevis* oocytes

Two-electrode voltage-clamp recordings were performed at room temperature (18–22 °C) by using a GeneClamp 500 amplifier (Molecular Devices, San Jose, CA, USA) controlled by a pClamp data acquisition system (Axon Instruments, Union City, CA, USA). Whole-cell currents from oocytes were recorded 1–4 days after mRNA injection. Bath solution composition was (in mM) as follows: 96 NaCl, 2 KCl, 1.8 CaCl₂, 2 MgCl₂ and 5 HEPES (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.8 and 1.5 M Ω . The elicited currents were filtered at 2 kHz and sampled at 20 kHz by using a 4-pole low-pass Bessel filter. Leak subtraction was performed by using a -P/4 protocol. From a holding potential of -90 mV with a start-to-start interval of 0.2 Hz. Na⁺ current traces were evoked by 100-ms depolarizations to V_{max} (the voltage corresponding to maximal Na⁺ current in control conditions). The current–voltage relationships were determined by 50 ms step depolarizations between -90 and 70 mV, using 5 mV increments.

All data are presented as mean $\pm$ SEM of at least five independent experiments ($n = 5$). All data were tested for normality by using a D'Agostino-Pearson omnibus normality test. All data were tested for variance by using the Bonferroni test or Dunn's test. Differences were considered significant if the probability that their difference stemmed from chance was 5% ($P < 0.05$). All data were analysed by using pClamp Clampfit 10.0 (Molecular Devices, San Jose, CA, USA) and Origin 7.5 software (OriginLab, Northampton, MA, USA).

2.11. TRPA1 FLIPR calcium assay

HEK293 cells heterologously expressing TRPA1 were plated at a density of 10,000–15,000 cells per well on black-walled 384-well imaging plates and cultured for 48 h. The cells were loaded with Calcium-4 no wash dye (Molecular Devices, San Jose, CA, USA) reconstituted to a working concentration with physiological salt solution (PSS; composition in mM: 140 NaCl, 11.5 glucose, 5.9 KCl, 1.4 MgCl₂, 1.2 NaH₂PO₄, 5 NaHCO₃, 1.8 CaCl₂, 10 HEPES, pH 7.4). Cells were incubated with 20 μ L of dye at 37 °C for 30 min. The cells were transferred to the FLIPR^{TETRA} fluorescence imaging platform where 50 μ M HSTX-I or matched DMSO buffer control was administered to the cells and incubated for 5 min. Cells were then stimulated with 300 μ M of the TRPA1 agonist allyl isothiocyanate (AITC) (Sigma Aldrich, St. Louis, MI, USA). Changes in fluorescence (excitation 470–495 nm; emission 515–575 nm) were measured at 1 s intervals for 770 s. Reads between 310 and 320 s were used as a baseline and the displayed trace was shown from 310 s (prior to AITC administration) until the end of the experiment.

2.12. Animals for behavioural studies

For *in vivo* experiments we used adult male C57BL/6J mice aged 8–10 weeks. Animals were housed in groups of 3 or 4 per cage, under 12 h light–dark cycles and had standard rodent chow and water ad libitum. Ethical approval for experiments involving animals was obtained from The University of Queensland animal ethics committees. All experiments were conducted in accordance with the *International Association for the Study of Pain Guidelines for the Use of Animals in Research and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, 8th edition (2013). Age-matched controls were used for studies involving knockout animals. Behavioural assessment was performed by a blinded observer unaware of the genotype and/or treatments received.

2.13. Generation of Nav1.9 knockout mice

Homozygous Nav1.9 knockout mice on C57BL/6J background were generated using CRISPR/Cas9 technology by the Queensland Facility for Advanced Genome Editing (The University of Queensland, Brisbane, Australia). In brief, two sgRNAs were designed to target exon 2 of the *Scn11a* gene. The deletion of exon 2 generated a frame shift mutation resulting in the introduction of stop codons in exon 3. The guide sgRNAs were microinjected together with Cas9 mRNA into fertilized eggs collected from C57BL/6J mice and transferred into pseudopregnant surrogate CD1 female mice. Offspring born to the foster mothers were genotyped by PCR (see below) and DNA sequencing. One F0 male mouse was bred to C57BL/6J female mice to generate F1 heterozygous mice, and F1 heterozygous mice were inbred to obtain homozygous mice. Genomic DNA was extracted from ear clippings and mice were genotyped by PCR using the following primers (forward: 5'-

GCTGCTC AGACACTCACAGT-3') and (reverse: 5' ATTCTGCCACCAGAGACTGC-3') by the Australian Equine Genetics Research Centre genotyping and sequencing facility (The University of Queensland, Brisbane, Australia).

2.14. Carrageenan model

Carrageenan (1% w/v) was administered by intraplantar injection in mice and mechanical and thermal allodynia was assessed 3 h post-injection as previously described [25]. HSTX-I (10 mg/kg), oxycodone (1 mg/kg), or saline (buffer control) was administered by intraperitoneal (i.p) injection (10 μ L/g) 10 min prior to assessment of mechanical and thermal thresholds. Mechanical thresholds were determined using electronic von Frey (MouseMet Electronic von Frey, TopCat Metrology, UK) as previously described [25]. Thermal thresholds were determined using the thermal probe test (MouseMet Thermal, TopCat Metrology, UK) as previously described [25].

3. Results

3.1. Synthesis and oxidation

Synthetic HSTX-I was produced based on the native peptide described by Wang et al. [13]. This included the disulfide connectivity (C2/C14 and C8/C19), which was originally determined by reductive alkylation and trypsin digest, and C-terminal amidation originally confirmed through carboxypeptidase Y treatment. Synthetic HSTX-I was assembled using automated solid phase peptide synthesis employing orthogonal cysteine sidechain protection in form of Trt on C2/C14, and AcM on C8/C19. Regioselective synthesis enabled stepwise folding of hydrophobic HSTX-I in organic solvents in high yield. Final yield was 70% based on crude peptide released from resin (Fig. 1A). Following each oxidation step, analytical RP-HPLC and ESI-LCMS was used to confirm successful formation of the disulfide bonds prior to proceeding with the next step. The same method was utilised to produce two alternative cystine connectivities (C2/C14 and C8/C19, C2/C19 and C8/C14; data not shown). 1D 1 H NMR spectra displayed well dispersed peaks in the amide region (7–10 ppm) suggesting that HSTX-I with C2/C14 and C8/C19 disulfide connectivity adopts a well-structured fold (Fig. 1C).

3.2. Synthetic HSTX-I shows limited activity at human Na_v channels

Native HSTX-I was originally described as a modestly potent (IC₅₀ ~ 3 μ M) blocker of rodent Na_v1.8 and Na_v1.9 currents by whole-cell patch-clamp electrophysiology in dissociated sensory neurons [16]. In our hands, when evaluated by whole-cell patch-clamp electrophysiology, synthetic HSTX-I (90 μ M due to limited peptide solubility) had no effect on heterologously expressed hNa_v1.7 currents (101 $\pm$ 3%; Fig. 2A), consistent with a lack of effect of native HSTX-I on TTX-s current at concentrations up to 100 μ M. However, synthetic HSTX-I did not inhibit heterologously expressed hNa_v1.8 at a concentration of 3 μ M (data not shown), and only reduced peak current by 20 $\pm$ 5.5% at the highest concentration tested (90 μ M; Fig. 2B). In addition, we tested the two alternative HSTX-I isomers (C2/C8 and C14/C19; C2/C19 and C8/C14) and observed no inhibition of hNa_v1.8 current up to a concentration of 10 μ M (C2/C8, C4/C19: 101 $\pm$ 13% control; C2/C19, C8/C14: 105 $\pm$ 9% control). Synthetic HSTX-I with the originally described C2/C14

and C8/C19 disulfide connectivity was also evaluated by voltage-clamp electrophysiology against hNav1.9 expressed in rat superior cervical ganglion (SCG) neurons, which lack endogenous TTX-r sodium channels and are thus a suitable model neuron for transfection of hNav1.9. Average peak hNav1.9 currents during baseline recordings was 1983 ± 1166 pA ($n = 4$). Application of HSTX-I ($10 \mu\text{M}$) decreased hNav1.9 currents to 1537 ± 747 pA ($n = 4$), which translates into a statistically significant block of $19.3 \pm 3.0\%$ of current (paired t -test, $p < 0.05$; Fig. 2C and D). Moreover, while inhibition of hNav1.9 current by synthetic HSTX-I was not reversible during washout (Fig. 2C), the observed effect was not due to current rundown as hNav1.9 currents were stable ($< 5\%$ change) over a 15 min period of baseline, mock treatment and washout recordings (Fig. 2E). HSTX-I was additionally tested against TTX-r channels hNav1.5, hNav1.8 and hNav1.9_C4 as well as hNav1.7 by two-electrode voltage-clamp recordings in *Xenopus laevis* oocytes, but no inhibition of current was observed under these conditions at peptide concentrations up to $20 \mu\text{M}$ (Fig. 2F).

3.3. Synthetic HSTX-I is not analgesic in a mouse model of carrageenan-induced inflammation

Native HSTX-I, dosed at up to 6.55 mg/kg by intraperitoneal injection, was previously reported to be anti-nociceptive in mouse models of formalin-induced spontaneous pain, acetic acid-induced writhing and the hot plate test [16]. We therefore sought to assess the analgesic effect of synthetic HSTX-I in a mouse model of carrageenan-induced inflammation, in which Nav1.9 has previously been shown to contribute to the development of mechanical and thermal allodynia [26]. Intraplantar administration of carrageenan caused a reduction in mechanical thresholds compared with baseline values (wt PWF: baseline $3.5 \pm 0.2 \text{ g}$; carrageenan $0.9 \pm 0.2 \text{ g}$) that was significantly attenuated in Nav1.9^{-/-} mice (Nav1.9^{-/-} PWF: baseline $3.3 \pm 0.1 \text{ g}$; carrageenan $2.2 \pm 0.2 \text{ g}$; Fig. 3A). However, despite previous activity in inflammatory pain models, as well as modest activity at Nav1.9, in our hands, administration of HSTX-I (10 mg/kg i.p.) had no significant effect on mechanical thresholds compared to buffer control, while the opioid receptor agonist oxycodone (1 mg/kg) significantly increased mechanical thresholds (PWF: control $1.3 \pm 0.2 \text{ g}$; HSTX-I $1.3 \pm 0.3 \text{ g}$; oxycodone $2.7 \pm 0.3 \text{ g}$, Fig. 3B). Similarly, carrageenan-induced heat allodynia was reduced in Nav1.9^{-/-} mice (PWT: wild type $43.2 \pm 0.5 \text{ }^{\circ}\text{C}$; Nav1.9^{-/-} $46.2 \pm 0.8 \text{ }^{\circ}\text{C}$; Fig. 3C), and in mice treated with oxycodone, while HSTX-I had no significant effect (PWT: control $44.6 \pm 0.7 \text{ }^{\circ}\text{C}$; HSTX-I $43.5 \pm 0.8 \text{ }^{\circ}\text{C}$; oxycodone $48.0 \pm 1.1 \text{ }^{\circ}\text{C}$; Fig. 3D).

3.4. NMR analysis of HSTX-I

We determined the NMR solution structure of synthetic HSTX-I to compare it to the model of native HSTX-I reported in the original paper, which predicted two β -strands between $\sim \text{F11-C14}$ and C19-V20 [13]. 2D TOCSY and NOESY spectra were obtained and used for sequential assignment of individual amino acid spin systems. To ascertain the presence of secondary structure within HSTX-I, secondary H α chemical shifts were determined by subtraction of random coil H α values from HSTX-I H α chemical shifts (Fig. 4A) [27]. Consecutive negative deviations at A1–C8 indicate an α -helix between these residues, whereas positive deviations between residues A10–C14 and I18–P21 indicate the presence of two antiparallel β -sheets. The secondary structure predicted arrangement of β -sheets in our NMR structure is consistent with the original model. Long-range NOEs and hydrogen

bonds between residues in this region further support these secondary structure observations (Fig. 4B).

3.5. Structure calculations of HSTX-I

The 3D structure of HSTX-I was calculated using a simulated annealing protocol based on 138 NOE-derived distance restraints, 17 Φ and 18 ψ dihedral restraints derived from TALOS-N (Table 1) [17]. χ_1 angle restraints were derived by DISH for C2, C8, C14 and C19 [28]. Additional restraints included disulfide connectivities (C2/C14 and C8/C19) and hydrogen bonds as determined from slow D₂O exchange (I18 $\rightarrow$ I15 and V20 $\rightarrow$ F13) and temperature coefficient experiments (I15 $\rightarrow$ I18). A total of 20 structures (Fig. 4C) with no NOE violations > 0.2 Å and no dihedral angle violations > 2 Å were chosen to represent HSTX-I based on lowest total energy and MolProbity score [20]. The final 20 structures were superimposed over the backbone heavy atoms between residues 2–19 and the mean pairwise RMSD was 1.19 ± 0.39 Å and 2.36 ± 0.47 Å for the backbone heavy atoms and all atoms, respectively. Energies and structural statistics for the NMR structures are shown in Table 1. The 20 structures with the lowest energies and best MolProbity scores were submitted to the Protein Data Bank and Biological Magnetic Resonance Bank (PDB ID: 6WQR, BMRB ID: 30750).

The NMR solution structure of HSTX-I is composed of an α -helix between residues Y5–G9 and two β -sheets between F13–I15 and I18–V20 (Fig. 4D). Medium range NOEs between residues Y5 and G9 support the observation of an α -helix, while hydrogen bonds (I15 $\rightarrow$ I18, I18 $\rightarrow$ I15, V20 $\rightarrow$ P13) supports the formation of the antiparallel β -sheets. HSTX-I is stabilised by two disulfide bonds between C2/C14 and C8/C19. Our calculated structure is in relative agreement with the original model, with the expectation of the α -helix in the N-terminal region.

Comparison of the calculated HSTX-I structure to the PDB identified structural similarities with Prototoxin-I (ProTx-I; PDB ID: 2M9L), a promiscuous Na_v channel inhibitor from tarantula-venom (Fig. 5A). The β -sheets in HSTX-I align closely to the β -sheets in ProTx-I, as well as one of the disulfide bonds. Interestingly, ProTx-I was also reported to inhibit transient receptor potential ankyrin 1 (TRPA1), a sensory nociceptor, which could provide an alternative target for HSTX-I [29].

3.6. Activity of HSTX-I at TRPA1

Given the structural similarity of HSTX-I to known TRPA1 inhibitors, we also tested HSTX-I against TRPA1 (Fig. 5A). However, HSTX-I at concentrations up to 15 μ M had no discernible effect at TRPA1, with the peptide neither eliciting Ca²⁺ influx nor modulating AITC-induced TRPA1 responses. At 50 μ M HSTX-I displayed a small inhibitory effect evidenced by a delay in the AITC-mediated calcium influx (Fig. 5B).

4. Discussion

Na_v1.8 and 1.9 have attracted considerable attention as putative analgesic targets based on their expression in small nociceptors as well as compelling functional and genetic data linking these sodium channel subtypes to pathological pain states. However, compared to

TTX-s subtypes such as Na_v1.7, relatively few subtype-selective modulators of these TTX-r isoforms have been reported [13,14].

Drug discovery efforts targeting Na_v1.9 in particular have suffered from long-standing difficulties with expression of functional channels in heterologous systems, which was recently attributed to a 49-residue motif in the C-terminal of Na_v1.9 that appears to regulate channel trafficking [30]. Accordingly, most studies have been restricted to recordings of native Na_v1.9 current from cultured rodent sensory neurons, or expression of human channels in suitable primary neurons. The need for these labour-intensive approaches have in turn limited pharmacological studies of Na_v1.9, with only few venom-derived Na_v1.9 modulators reported to date. These include TsVII and ProTx-I – peptides isolated from the venom of the scorpion *Tityus serrulatus* and the tarantula *Thrixopelma pruriens*, respectively – which enhance rather than block Na_v1.9 currents [15]. Similarly, although heterologous expression of Na_v1.8 has facilitated the development of small molecule modulators such as PF-01247324 [31] and A-803467 [32], peptide toxins with activity at this subtype are comparatively rare. Examples of venom-derived Na_v1.8 modulators include the family of μ O-conotoxins [33–35], as well as more recently described peptides from *Naja atra* venom (μ -EPTX-Na1a) [14], *Haplopelma lividum* venom (μ -TRTX-H11a) [13] and CcoTx1, CcoTx2, and CcoTx3 from the *Ceratogyrus cornuatus* tarantula [36]. Interestingly, native HSTX-I was the first reported sodium channel modulator isolated from leech saliva, and was particularly notable for its activity at both Na_v1.8 and Na_v1.9, which is rare given that these isoforms only share ~ 48% amino acid sequence identity. As these studies were carried out with purified rather than synthetic material, and contamination with minor bioactive components is always a possibility, we sought to synthesise HSTX-I and confirm activity in heterologous expression systems.

To produce synthetic HSTX-I we developed a one-pot oxidation protocol and single step RP-HPLC purification protocol which permitted rapid production of the amidated peptide in high yield, displaying the disulfide connectivity suggested by Wang et al. [13]. We were able to confirm lack of activity of synthetic HSTX-I at Na_v1.7. However, in our hands the peptide did not substantially inhibit Na_v1.8 at the highest concentration (90 μ M) we were able to test due to limited solubility of the peptide, whilst the original report stated an IC₅₀ of 2.44 μ M against Na_v1.8. Moreover, while the original paper reported an IC₅₀ of ~ 3 μ M for HSTX-I at Na_v1.9 in voltage-clamp recordings, our synthetic peptide was only very modestly active at heterologously expressed hNa_v1.9, with 10 μ M synthetic HSTX-I reducing peak current size by ~ 19%. Thus, the synthetically produced HSTX-I was considerably less potent than the reported activity of native peptide at endogenous Na_v currents from rodent dorsal root ganglion neurons (IC₅₀ ~ 3 μ M). Several factors may contribute to these discrepancies. Synthetic HSTX-I was folded according to the experimentally determined disulfide connectivity and produced with a C-terminal amide. Thus, while we were unable to confirm co-elution with the native product, it is unlikely – though not impossible – that our synthetic peptide lacked activity due to misfolding. To investigate this we produced HSTX-I analogues with the two alternative disulfide connectivities. However, these also lacked activity at Na_v1.8 when tested up to 10 μ M. It is notable that the native purified fraction used for activity testing in the original study contained several masses that could be attributed to sodium and potassium adducts, as well

as several unrelated minor components that may have contributed to inhibition of native Na_V currents in rodent neurons. It is also possible that cell background contributed to the lack of activity of synthetic HSTX-I, although we observed a loss of activity across several expression systems including HEK293 and CHO cells, *Xenopus* oocytes, as well as SCG neurons. Notably, HSTX-I also lacked activity in a murine model of inflammatory pain despite $\text{Na}_V1.9^{-/-}$ mice showing clear attenuation of both mechanical and thermal allodynia. It thus seems unlikely that synthetic HSTX-I is considerably more active at $\text{mNa}_V1.9$ than $\text{hNa}_V1.9$. In light of previously reported efficacy of lower doses of native HSTX-I, peptide stability or insufficient peptide exposure are also unlikely to contribute to lack of *in vivo* activity. Future studies will be required to determine whether synthetic HSTX-I would be analgesic in nociceptive models such as formalin-induced pain or the hot plate test.

We determined the NMR solution structure of synthetic HSTX-I using 2D homonuclear NMR. HSTX-I is highly structured and composed an α -helix between Y5–G9 and two antiparallel β -sheets between F13–I15 and I18–V20, connected by a type-I β -turn. In the original study HSTX-I structure was modelled against AmFPI-1, a fungal protease inhibitor from *Antheraea mylitta*, selected by BLAST results and predicted to contain two β -sheets. Our NMR calculations were in relative agreement with this model; however, we showed the addition of a short N-terminal α -helix. Interestingly, despite not sharing sequence similarities, structural similarities were observed between HSTX-I and ProTx-I. In addition to ProTx-I displaying activity at $\text{Na}_V1.9$, this peptide is also a known inhibitor of TRPA1, another sensory ion channel. We therefore investigated HSTX-I ability to inhibit TRPA1 as a possible mechanism for the reported antinociception. HSTX-I lacked activity against TRPA1 at concentrations up to 15 μM . However, at 50 μM , HSTX-I caused a small delay of AITC-mediated calcium influx. Given that formalin-induced pain is mediated by TRPA1 activation [37], it is possible that this activity could account for the analgesic response reported in the original study, and could be investigated in future studies.

In conclusion, we developed a one-pot oxidation and single purification peptide synthesis protocol to produce correctly folded synthetic HSTX-I, which lacked potent activity at heterologously expressed $\text{Na}_V1.8$ or $\text{Na}_V1.9$ and was not analgesic in a murine model of inflammatory pain. These results suggest that this leech-derived peptide is not a useful lead for development of analgesic $\text{Na}_V1.8$ and 1.9 modulators.

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Fig. 1. Synthesis and oxidation of HSTX-I. **A** Schematic of HSTX-I oxidation protocol. **B** RP-HPLC and ESI-LCMS spectra (insert) after first cysteines were deprotected and oxidised (left) and RP-HPLC and ESI-LCMS spectra after removal of Acm protecting group (Mw 72), second cysteines oxidised and peptide purified (right). **C** 1D ^1H NMR spectra of HSTX-I shows well dispersed peaks in the amide region.

Fig. 2.

HSTX-I inhibition of human Na_v currents. **A** HSTX-I (90 μ M) did not reduce peak currents in Na_v1.7 expressing HEK93 cells, but **B** reduced peak current by a mean 20% in Na_v1.8 expressing CHO cells at a concentration of 90 μ M. **C** Voltage-clamp traces at Na_v1.9 for baseline (black), HSTX-I (10 μ M; red) and washout (green) periods. SCG neurons were held at -100 mV and stepped to -45 mV for 50 ms from a conditioning pulse of -120 mV for 1 sec. Depolarizing pulses were applied every 30 s until stable current responses were achieved. **D** Na_v1.9 currents are blocked by HSTX-I at the 10 μ M concentration by

19.3 ± 3.0% (n = 4), * $P < 0.05$ compared to baseline. **E** Stable $\text{Na}_V1.9$ current during mock treatment with vehicle alone over 15 min period. **F** Superimposed current traces for the indicated Nav isoforms before (black) and after application of 20 μM HSTX-I (blue). Currents were elicited by depolarizing pulses to 0 mV. The asterisk indicates the peak current level in the presence of toxin; the dotted line indicates 0 current level.

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Fig. 3.

Effect of HSTX-I in a mouse model of carrageenan-induced inflammation. **A** Mechanical threshold of wild type and Nav1.9^{-/-} mice following intraplantar injection of carrageenan. **B** Effect of HSTX-I (10 mg/kg i.p) and oxycodone (1 mg/kg i.p) on mechanical thresholds following intraplantar injection of carrageenan. **C** Heat thresholds of WT and Nav1.9^{-/-} mice following intraplantar injection of carrageenan. **D** Effect of HSTX-I (10 mg/kg i.p) and oxycodone (1 mg/kg i.p) on heat thresholds following intraplantar injection of carrageenan. Data are presented as mean ± SEM. Statistical significance was determined using unpaired *t*-test or one-way ANOVA with Dunnett's post-test as appropriate, **P* < 0.05 compared to wild type or control.

Fig. 4.

NMR structural determination of HSTX-I. **A** Secondary H_{α} chemical shift deviations from random coil values calculated from 2D TOCSY NMR spectra. The structure was predicted to have an α -helix between residues A1–C8 (red helix) and two β -sheets between residues A10–C14 and I18–P21 (blue arrows). **B** Representation of the secondary structure antiparallel β -sheets in HSTX-I. Double headed arrows indicated observed NOEs and dashed lines represent putative hydrogen bonds and single-headed arrows represents a type I β -turn. Summary of local and medium range NMR data of HSTX-I with bar width representing the strength of the NOE. Open circles indicate slow exchange NH protons ($> 4\text{h}$). The α -helix and β -sheet regions are denoted by curved line and arrows, respectively. **C** The 20 lowest energy conformations of NMR solution structure as analysed by MOLMOL superimposed across C2 and C19. **D** The final 3D structure of HSTX-I following refinement

in CNS is composed of a single α -helix between residues Y5–G9 (red helix) and two antiparallel β -sheet F13–I15 and I18–V20 (blue arrows), stabilised by two disulfide bridges (yellow).

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Fig. 5. Effect of HSTX-I against TRPA1. **A** NMR solution structure of HSTX-I compared to ProTx-I highlight structural similarities including alignment of β -sheets and disulfide bond (sticks) **B** 50 μ M HSTX-I (blue circles) induces a delayed response compared to the DMSO buffer control (red squares) on TRPA1-expressing cells following 300 μ M AITC-mediated calcium influx.

Table 1

Statistical analysis of HSTX-I NMR solution structure.

Distance restraints	
Intraresidue ($i-j = 0$)	65
Sequential ($ i-j = 1$)	51
Medium range ($ i-j < 5$)	7
Long range ($ i-j > 5$)	7
Hydrogen bonds ^a	8
Total	138
Dihedral angle restraints	
Φ	17
Ψ	18
χ^1	4
Total	39
Structure statistics	
Energies (kcal/mol, mean $\pm$ SD)	
Overall	-667.6 ± 42.5
Bonds	11.3 ± 1.1
Angles	29.6 ± 3.9
Improper	9.8 ± 2.5
Dihedral	99.5 ± 1.6
Van de Waals	-75.1 ± 5.2
Electrostatic	-743.1 ± 45.6
NOE (experimental)	0.1 ± 0.02
Constrained dihedrals (experimental)	0.3 ± 0.2
Atomic RMSD ($\text{\AA}$)	
Mean global backbone (2–19) ^b	1.19 ± 0.39
Mean global heavy (2–19) ^b	2.36 ± 0.47
Mean global backbone (1–23)	1.66 ± 0.41
Mean global heavy (1–23)	2.64 ± 0.45
MolProbity statistics	
Clash score, all atoms ^c	5.4 ± 3.9
Poor rotamers	0.2 ± 0.4
Ramachandran outliers (%)	1.6 ± 2.3
Ramachandran favoured (%)	95.0 ± 3.5
MolProbity score	1.6 ± 0.5
MolProbity percentile ^d	85.3 ± 14.9
Violations	
Distance constraints ($> 0.2 \text{ \AA}$)	0
Dihedral-angle constraints ($> 2^\circ$)	0

^aTwo hydrogen bond restraints included per bond.

^bPairwise RMSD from 20 refined structures over amino acids 2–19.

^cNumber of steric overlaps ($> 0.4 \text{ \AA}$)/1000 atoms.

^d100% is the best among structures of comparable resolution. 0% is the worst.

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