Manipulation of a spider peptide toxin alters its affinity for lipid bilayers and potency and selectivity for voltage-gated sodium channel subtype 1.7

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Huwentoxin-IV (HwTx-IV) is a gating modifier peptide toxin from spiders that has weak affinity for the lipid bilayer. As some gating modifier toxins have affinity for model lipid bilayers, a tripartite relationship among gating modifier toxins, voltage-gated ion channels, and the lipid membrane surrounding the channels has been proposed. We previously designed an HwTx-IV analogue (gHwTx-IV) with reduced negative charge and increased hydrophobic surface profile, which displays increased lipid bilayer affinity and in vitro activity at the voltage-gated sodium channel subtype 1.7 (NaV1.7), a channel targeted in pain management. Here, we show that replacements of the positively-charged residues that contribute to the activity of the peptide can improve gHwTx-IV’s potency and selectivity for NaV1.7. Using HwTx-IV, gHwTx-IV, [R26A]gHwTx-IV, [K27A]gHwTx-IV, and [R29A]gHwTx-IV variants, we examined their potency and selectivity at human NaV1.7 and their affinity for the lipid bilayer. [R26A]gHwTx-IV consistently displayed the most improved potency and selectivity for NaV1.7, examined alongside off-target NaV,s, compared with HwTx-IV and gHwTx-IV. The lipid affinity of each of the three novel analogues was weaker than that of gHwTx-IV, but stronger than that of HwTx-IV, suggesting a possible relationship between in vitro potency at NaV1.7 and affinity for lipid bilayers. A murine NaV1.7 engagement model, [R26A]gHwTx-IV exhibited an efficacy comparable with that of native HwTx-IV. In summary, this study reports the development of an HwTx-IV analogue with improved in vitro selectivity for the pain target NaV1.7 and with an in vivo efficacy similar to that of native HwTx-IV.

Voltage-gated sodium channels (NaVs)3 are involved in almost all aspects of human physiology, in particular in initiation and propagation of neurotransmission. To date, nine NaVs subtypes (NaV1.1–1.9) have been described based on their distribution and physiological function (1), with loss- and gain-of-function mutations in individual subtypes resulting in a number of pathophysiological conditions, including pain (2, 3), epilepsy (4, 5), and neuromuscular (6, 7) and cardiac disorders (8). More specifically, NaV1.7–1.9 are being pursued as targets for pain therapeutics because of their involvement in pain-related pathologies, including neuropathic pain (3, 9–11), mechanical pain associated with inflammatory bowel disease (12), diabetic neuropathy (13–15), and inflammation (16, 17). The NaV architecture is typified by four domains (domains I–IV), connected via intracellular loops of various lengths, with each domain containing six segments (S) such that S1–S4 form the voltage sensor domains, and S5 and S6 form the pore domain (Fig. 1A) (18). Because of high-sequence homology between subtypes NaV1.1–1.7 (NaV1.8 and NaV1.9 display low-sequence homology across known binding sites) (Fig. 1C) (19), only highly-specific inhibitors would be of interest in order to avoid unwanted off-target effects.

In the search for novel modulators of these channels, gating modifier toxins (GMTs) discovered in spider venom have proven to be useful pharmacological probes and drug leads for conditions mediated by NaV function due to their high specificity and selectivity for the therapeutically-relevant sodium channel subtype NaV1.7 (20–25). Most spider GMT’s are disulfide-rich and fold to adopt a knottin motif consisting of disulfide bridges formed between Cys I–IV, Cys II–V, and Cys

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3 The abbreviations used are: NaV, voltage-gated sodium channel; GMT, gating modifier toxin; FLIPR, fluorescence imaging plate reader; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; SPR, surface plasmon resonance; TIPS, triisopropylsilane; PDB, Protein Data Bank; RP-HPLC, reverse-phase HPLC; Acm, acetamidomethyl; 4-MeBzl, 4-methylbenzyl; Trt, S-trityl; ACN, acetonitrile; tBu, t-buty1; Boc, t-butoxycarbonyl; HwTx-IV, huwentoxin-IV; ProTx-II, protoxin-II; HF, hydrogen fluoride; PSS, physiological salt solution; P/L, peptide/lipid.
III–VI (26, 27). GMTs also share a hydrophobic patch and charged ring on their surface profiles (Fig. 1B), reminiscent of membrane-interacting peptides (28–31).

As some GMTs bind to lipid bilayers (28–30), the concept of a three-way interaction between GMTs, voltage-gated ion channels, and the lipid membrane has arisen (28, 30, 32–35). Using protoxin-II (ProTx-II), a GMT with affinity for lipid membranes (36, 37), we recently showed that reducing the lipid affinity affects selectivity for NaV1.7. GMT selectivity for the channels for improved potency (33, 34, 37, 39–42).

The same study also hypothesized that Lys-27 played an important role in the bioactivity of HwTx-IV (49). Additional studies by Minassian et al. (48) showed that R26A and K27A mutations of HwTx-IV resulted in some loss of potency at NaV1.7, accompanied by larger reductions in potency at NaV1.2. The R29A mutation resulted in no change in potency at NaV1.7, but a 260-fold of activity at tetrodotoxin-sensitive NaVs, including NaV1.8 (examined on dorsal root ganglion cells) (49).

Previous mutagenesis studies have deduced that the positively-charged amino acids on loop 4 of HwTx-IV are of particular importance for activity (Fig. 1B) (24, 48, 49). Deng et al. (49) noted that among other residues on loop 4, the R29A mutant lost ~60% of activity at tetrodotoxin-sensitive NaV1.7, including NaV1.8 (examined on dorsal root ganglion cells) (49). The same study also hypothesized that Lys-27 played an important role in the bioactivity of HwTx-IV (49). Additional studies by Minassian et al. (48) showed that R26A and K27A mutations of HwTx-IV resulted in some loss of potency at NaV1.7, accompanied by larger reductions in potency at NaV1.2. The R29A mutation resulted in no change in potency at NaV1.7, but a considerable reduction in potency at NaV1.2 (these studies were conducted on NaV1.8 expressed in HEK293 cells) (48). The main difference between NaV1.7 and NaV1.2 at the putative GMT-binding site on S3 and S4 of DII is that NaV1.2 only has two anionic residues compared with three on NaV1.7 in the putative GMT-binding site (NaV1.2 contains Asn-816 in place of Asp-816) (Fig. 1C). Therefore, the reduction in potency for the Arg-29 analogue could be due to weakened electrostatic interactions between HwTx-IV and NaV1.2 (46). Recent studies by Tzakoniati et al. (50) and Neff et al. (51) propose that interactions between anionic residues on both the S1 and S2 loop and S3 and S4 loop and cationic residues Arg-26, Arg-29, and...
Lys-27 are important in HwTx-IV binding (50, 51), although there is some disagreement on the specific interactions across existing models (48, 50, 51). Based on these observations, we hypothesized that manipulation of the basic residues on loop 4 of HwTx-IV may aid in improving selectivity by modulating salt-bridge interactions between basic amino acids on loop 4 of the GMT and anionic amino acids in the conserved S3 and S4 extracellular loop of DII on off-target NaV, s.

We used gHwTx-IV (24, 34) as a starting molecule to capitalize on the increased potency of this molecule at NaV, 1.7, which is accompanied by stronger affinity for the lipid bilayer compared with HwTx-IV. We previously postulated that the lipid bilayer can attract and concentrate gHwTx-IV near its binding site at NaV, 1.7 to increase its apparent potency (34). Even though Arg-26, Lys-27, and Arg-29 mutations on HwTx-IV did not always display favorable potency at NaV, 1.7 (48, 49), we were interested in examining whether point mutations to Arg-26, Lys-27, and Arg-29 of gHwTx-IV would improve selectivity while maintaining potency at NaV, 1.7 through interactions with the lipid bilayer. To this end, we synthetically produced HwTx-IV, gHwTx-IV, [R26A]gHwTx-IV, [K27A]gHwTx-IV, and [R29A]gHwTx-IV (Fig. 1C). We ensured correct folding of the analogues using NMR, examined lipid binding of the peptides using surface plasmon resonance (SPR), and the activity of all peptides on human NaV, 1.7 was assessed using automated patch-clamp electrophysiology. In addition, we were specifically interested in examining selectivity for NaV, 1.7 over off-target NaV, s that possess high-sequence homology to NaV, 1.7 at the putative binding site for HwTx-IV; therefore, peptides were tested on human NaV, 1.1, NaV, 1.2, NaV, 1.3, NaV, 1.4, NaV, 1.5, and NaV, 1.6 for off-target activity (Fig. 1C). To examine the ability of HwTx-IV and the analogues to inhibit activity of the channels, the most potent analogue at NaV, 1.7 was determined using automated patch-clamp electrophysiology, and its potency at NaV, 1.1–1.7 was subsequently determined. The NaV, 1.1–1.7 selectivity profiles for HwTx-IV and all analogues were determined using a membrane potential assay on FLIPRTETRA (52). Taken together, this body of work demonstrates that for gHwTx-IV, mutations to amino acids involved in activity can improve selectivity for NaV, 1.7 and that the same modifications decrease affinity for lipid bilayers. The most potent analogue, [R26A]gHwTx-IV, was further tested for on-target activity in vivo using a mouse model of NaV, 1.7-mediated nociception, and it showed improved efficacy compared with gHwTx-IV and similar efficacy to HwTx-IV.

Results

Oxidation and structural analysis of the peptides

To attain correctly folded peptides, oxidation of HwTx-IV was conducted over 16 h to obtain the oxidized peptide with the most thermodynamically favorable fold and a yield of 80% as described previously (Fig. S1) (28, 34, 46). Thermodynamic oxidation also facilitated the folding of gHwTx-IV, [R26A]gHwTx-IV, and [K27A]gHwTx-IV with yields between 10 and 15% over 48 h (Fig. S1) (24, 34). However, repeated misfolding of [R29A]gHwTx-IV using thermodynamic approaches prompted the use of orthogonal oxidation (Fig. 2 and Figs. S1 and S2).

[R29A]gHwTx-IV was synthesized with S-trityl (Trt) protecting groups on Cys III and Cys VI were oxidized slowly in DMSO, followed by a fast removal of Acm protecting groups with subsequent oxidation of Cys II and Cys V using I2. After HF cleavage of 4-MeBzl, Cys I and Cys IV were oxidized slowly in DMSO to give the final product with correct disulfide connectivity. MALDI-TOF was used to ascertain that the correct product was formed at each step, and RP-HPLC analytical traces show a leftward shift in retention time after each oxidation step.
Expected local differences in the backbones of the peptides are observed between gHwTx-IV and HwTx-IV at the E1G, E4G, F6W, and Y33W mutations as before (34), and between gHwTx-IV and the three analogues at R26A, K27A, and R29A (Fig. 3).

Affinity of GMTs for lipid bilayers

SPR was used to examine the affinity of the GMTs for lipid bilayers by measuring the amount of peptide bound to lipid (peptide/lipid (P/L mol/mol)) using zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and anionic POPC/POPS (4:1 molar ratio) model membranes. The rationale behind the choice of lipids for these studies has previously been discussed (28, 33, 34, 37, 53). Briefly, phospholipids containing phosphatidylcholine headgroups are abundant on the outer leaflet of mammalian cells, and phospholipids containing phosphatidylserine headgroups mimic the anionic environment surrounding membrane-embedded voltage-gated sodium channels (54).

As before (34), HwTx-IV had weak affinity for model lipid bilayers (all values are maximum P/L mol/mol/H11006 ± S.E.) compared with gHwTx-IV (POPC = 0.07 ± 0.02; POPC/POPS = 0.16 ± 0.03) (Fig. 4). [R26A]gHwTx-IV (POPC = 0.024 ± 0.003; POPC/POPS = 0.06 ± 0.01), [K27A]gHwTx-IV (POPC = 0.06 ± 0.01; POPC/POPS = 0.12 ± 0.01), and [R29A]gHwTx-IV (POPC = 0.05 ± 0.01; POPC/POPS = 0.06 ± 0.01) had intermediate affinities for the lipid bilayers compared with gHwTx-IV and HwTx-IV. We previously described the membrane-binding face of gHwTx-IV to include Gly-1, Gly-4, Trp-6, and Trp-33 among other residues on the hydrophobic patch (34). The results herein suggest that the positively-charged amino acid residues on loop 4 of the GMT also contribute to affinity for lipid bilayers and that substitution of these residues for alanine residues on gHwTx-IV reduces their affinity for lipid bilayers.

Potency and selectivity of the GMTs

Potency at human Na\textsubscript{v}1.7 was improved by each of the mutations compared with HwTx-IV, as determined by automated patch-clamp electrophysiology (Fig. 5A and Fig. S3A). As before (34), gHwTx-IV was ~4-fold more potent than HwTx-IV (HwTx-IV IC\textsubscript{s50} = 35.7 ± 5.2 nM; gHwTx-IV IC\textsubscript{s50} = 8.1 ± 0.3 nM). Potencies of [K27A]gHwTx-IV and [R29A]gHwTx-IV were comparable with gHwTx-IV ([K27A]gHwTx-IV IC\textsubscript{s50} = 8.5 ± 2.0 nM; [R29A]gHwTx-IV IC\textsubscript{s50} = 7.7 ± 2.6 nM). Interest-
ingly, [R26A]gHwTx-IV was nearly five times more potent than gHwTx-IV and 21-fold more potent than HwTx-IV at NaV1.7 (IC50 = 0.5 nM) (Fig. 5A and Table 1).

Selectivity of [R26A]gHwTx-IV for human NaV1−1.6 was then determined by automated patch-clamp electrophysiology (Fig. 5B and Fig. S3B). Although NaV1.8 and NaV1.9 are both validated pain targets (1, 11, 16, 17), these subtypes were not included in this study as they have low sequence homology across the proposed binding site compared with NaV1.7 (19). Furthermore, NaV1.9 is notoriously challenging to express and is only available in a handful of laboratories in the world (55, 56). [R26A]gHwTx-IV showed 11- and 13-fold selectivity for NaV1.7 compared with NaV1.6 and NaV1.1, respectively. Selectivity was 27-fold greater at NaV1.2 and 64-fold greater at NaV1.3. The peptide showed no activity at NaV1.4 or NaV1.5 up to 300 nM; therefore, selectivity at both subtypes is at least 176-fold (Fig. 5B and Table 2). This results in an [R26A]gHwTx-IV selectivity profile of NaV1.7 > NaV1.6 > NaV1.1 > NaV1.2 > NaV1.3 > NaV1.4 and NaV1.5.

Time constants of block (τ) were determined using automated patch-clamp electrophysiology for HwTx-IV, gHwTx-IV, and the most potent analogue at NaV1.7, [R26A]gHwTx-IV (Fig. 5C). Peptides at their respective IC90 concentrations (HwTx-IV, 100 nM; gHwTx-IV, 20 nM; [R26A]gHwTx-IV, 10 nM) were added to cells to achieve near-complete block, and the decrease in current was measured over a period of 20 min, during which all peptides reached a steady state. It is necessary to test each peptide at its IC90 because τ values are concentration-dependent.

Table 1

| Peptide            | IC90 ± S.E. (nM) |
|--------------------|------------------|
| HwTx-IV            | 35.7 ± 5.2       |
| gHwTx-IV           | 8.1 ± 0.3        |
| [R26A]gHwTx-IV     | 1.7 ± 0.5        |
| [K27A]gHwTx-IV     | 8.5 ± 2.0        |
| [R29A]gHwTx-IV     | 7.7 ± 2.6        |

Table 5. Potency of HwTx-IV, gHwTx-IV, and analogues on NaV1.7

Data were measured using automated patch-clamp electrophysiology on HEK293 cells, n ≥ 3.
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Table 2

Potency and selectivity of [R26A]gHwTx-IV on Na\(_{\text{v}}\).1,1-1.7

| Subtype   | IC\(_{50}\) ± S.E. | Selectivity |
|-----------|--------------------|-------------|
| Na\(_{\text{v}}\).1 | 22.4 ± 1.2 | 13.2 |
| Na\(_{\text{v}}\).1.2 | 45.6 ± 1.2 | 26.8 |
| Na\(_{\text{v}}\).1.3 | 109.3 ± 48.2 | 64.3 |
| Na\(_{\text{v}}\).1.4 | >300 | >176 |
| Na\(_{\text{v}}\).1.5 | >300 | >176 |
| Na\(_{\text{v}}\).1.6 | 18.7 ± 4.3 | 11.0 |
| Na\(_{\text{v}}\).1.7 | 1.7 ± 0.5 | |

This is similar to the remaining inhibition of HwTx-IV lower percentage of remaining inhibition (28.7\%) effect on peptide dissociation. the highest percentage of sustained inhibition (68.1\%) (Fig. 5C). After continuous wash-off for 30 min, gHwTx-IV showed activity. Fold Na\(_{\text{v}}\).1.7 selectivity is IC\(_{50}\) Na\(_{\text{v}}\).1.x/IC\(_{50}\) Na\(_{\text{v}}\).1.7. [R26A]gHwTx-IV and [R29A]gHwTx-IV have some effect on the rate of binding to the channel. Time constants of block were also measured for the same three peptides at 100 nm to compare them at the same concentration (Fig. 5D), which showed similar \(\tau\) values for all peptides (HwTx-IV, \(\tau = 0.753\) s; gHwTx-IV, \(\tau = 1.48\) s; and [R26A]gHwTx-IV, \(\tau = 1.30\) s).

A stepwise wash-off was performed with HwTx-IV, gHwTx-IV, and [R26A]gHwTx-IV at their respective IC\(_{50}\) values (Fig. 5C). After continuous wash-off for 30 min, gHwTx-IV showed the highest percentage of sustained inhibition (68.1\% ± 7.6\%). This is similar to the remaining inhibition of HwTx-IV (52.1\% ± 13.3\%), but [R26A]gHwTx-IV had a significantly lower percentage of remaining inhibition (28.7\% ± 8.8\%). This suggests that the mutations that make up HwTx-IV do not affect the peptide's dissociation from the channel compared with HwTx-IV, whereas the R26A mutation has a significant effect on peptide dissociation.

IC\(_{50}\) values of the same three peptides were measured with holding potentials of both −90 mV and −55 mV to determine whether binding is state- or voltage-dependent, showing no significant difference (Fig. S4). This suggests that although there is strong evidence that HwTx-IV binds preferentially to the closed conformation of voltage-sensor DII, the modified toxins could experience some alterations in binding to the activated voltage-sensor, and unlike for some other GMTs (57, 58), the activity of the peptides tested in this study appear not to be influenced by channel state or membrane potential.

A complete selectivity screen was performed for each peptide using the FLIPR\({\text{TETRA}}\) membrane potential assay (Table 3, Fig. 6). The addition of veratridine is known to underestimate the potency of GMTs compared with electrophysiology measurements, as reported previously (59). Although this assay gives a median 15-fold underestimation of the potencies of GMTs compared with patch-clamp electrophysiology assays (as observed in this study) (Figs. S5 and S6 and Table S1), it is a lower-cost, high-throughput assay that can reliably be used to measure relative potencies and therefore selectivity. Overall, the observed trend of selectivity for Na\(_{\text{v}}\).1.7 using both assays is in good agreement.

From the membrane potential assay, selectivity of [R26A]gHwTx-IV and [K27A]gHwTx-IV was 9-fold (comparable with 13-fold for [R26A]gHwTx-IV, as determined by patch-clamp electrophysiology), and [R29A]gHwTx-IV was 54-fold more selective for Na\(_{\text{v}}\).1.7 than Na\(_{\text{v}}\).1.1 (Tables 2 and 3). This was an improvement compared with the selectivity of HwTx-IV (3-fold) and HwTx-IV (less than 1-fold) (Table 3). Similarly, [K27A]gHwTx-IV and [R29A]gHwTx-IV showed a 12- and 13-fold selectivity for Na\(_{\text{v}}\).1.7 over Na\(_{\text{v}}\).1.2 respectively (Table 3). The selectivity of [R26A]gHwTx-IV was the most improved at 41-fold (overestimated compared with 27-fold, as determined by patch-clamp electrophysiology) (Tables 2 and 3). gHwTx-IV had 1.5-fold selectivity for Na\(_{\text{v}}\).1.7 and Na\(_{\text{v}}\).1.2, and HwTx-IV showed almost equivalent potency for both Na\(_{\text{v}}\).1.7 and Na\(_{\text{v}}\).1.2 (Table 3). [R26A]gHwTx-IV and [R29A]gHwTx-IV had a 92-fold (overestimated compared with 64-fold from patch-clamp electrophysiology) and 273-fold selectivity (respectively) for Na\(_{\text{v}}\).1.7 compared with Na\(_{\text{v}}\).1.3 (Tables 2 and 3). In this case, the selectivity of [K27A]gHwTx-IV was 11-fold, which was less than the selectivity of HwTx-IV (83-fold). HwTx-IV did not show inhibitory activity at Na\(_{\text{v}}\).1.3 up to 10 \(\mu\)M in our hands, making it at least 41-fold more selective for Na\(_{\text{v}}\).1.7 over Na\(_{\text{v}}\).1.3 (Table 3), although an IC\(_{50}\) value of 338 nM was previously reported at rat Na\(_{\text{v}}\).1.3 (46). In comparison with Na\(_{\text{v}}\).1.7 to Na\(_{\text{v}}\).1.4, [R26A]gHwTx-IV showed the greatest improvement in selectivity for Na\(_{\text{v}}\).1.7, showing no inhibitory activity on Na\(_{\text{v}}\).1.4 up to 10 \(\mu\)M, thus being at least 392-fold more selective (or at least 176-fold, from patch-clamp electrophysiology) (Tables 2 and 3). [R29A]gHwTx-IV was 309-fold more selective and [K27A]gHwTx-IV was 236-fold more selective for Na\(_{\text{v}}\).1.7 than Na\(_{\text{v}}\).1.4 (Table 3). By comparison, gHwTx-IV was 324-fold more selective and HwTx-IV was 41-fold more selective for Na\(_{\text{v}}\).1.7 than for Na\(_{\text{v}}\).1.4 (Table 3).

None of the peptides showed activity at Na\(_{\text{v}}\).1.5; therefore, if IC\(_{50}\) values were set at an arbitrary value of 10 \(\mu\)M (Fig. 6), the selectivity of the peptides relative to Na\(_{\text{v}}\).1.7 in ascending order is HwTx-IV (41-fold), [K27A]gHwTx-IV (50-fold), gHwTx-IV (136-fold), [R29A]gHwTx-IV (187-fold), and [R26A]gHwTx-IV (392-fold) (Table 3). Compared with Na\(_{\text{v}}\).1.6, [R26A]gHwTx-IV and [R29A]gHwTx-IV were 56-fold (overestimated compared with 11-fold, determined by patch-clamp electrophysiology) and 55-fold more selective for Na\(_{\text{v}}\).1.7, and [K27A]gHwTx-IV was 22-fold more selective for Na\(_{\text{v}}\).1.7 over Na\(_{\text{v}}\).1.6. gHwTx-IV and HwTx-IV had 9- and 24-fold selectivity, respectively, for Na\(_{\text{v}}\).1.7 (Table 3).

These results show that a combination of mutations to HwTx-IV, including gHwTx-IV (E1G, E4G, F6W, and Y33W), and point mutations at the positively-charged residues in loop 4 lead to modified potency and selectivity of this GMT at Na\(_{\text{v}}\).1.7.

Taking these results together with the GMT–lipid affinity studies, removal of positively-charged residues that contribute to the activity of HwTx-IV reduces, but does not eliminate, affinity for the lipid bilayer. These mutations also improve the overall selectivity for Na\(_{\text{v}}\).1.7 with respect to off-target Na\(_{\text{v}}\).s containing a string of anionic residues at HwTx-IV’s putative binding site (Fig. 1, C and D).
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Activity of HwTx-IV, gHwTx-IV, and [R26A]gHwTx-IV in murine model of Na\(_{\text{v}}\).1.7-mediated nociception

We were interested in examining whether the most selective peptide was efficacious \textit{in vivo}; therefore, the effects of HwTx-IV, gHwTx-IV, and [R26A]gHwTx-IV were examined in a mouse model of Na\(_{\text{v}}\).1.7-mediated nociception after intraplantar administration of OD1 (300 nm), an α-scorpion toxin that selectively impairs inactivation and enhances current from Na\(_{\text{v}}\).1.7 (Fig. 7) (52, 60). There is 100% homology between human and mouse Na\(_{\text{v}}\).1.7 across the S3 and S4 helix sequence in D1II, which includes the S3 and S4 extracellular loop previously described as the GMT-binding site, suggesting that a mouse model provides a suitable comparison for human Na\(_{\text{v}}\).1.7 (UniProt ID Q15858 hNa\(_{\text{v}}\).1.7 and Q62205 mNa\(_{\text{v}}\).1.7) (Fig. S7). HwTx-IV significantly reduced nocifensive behaviors (cumulative nocifensive behavior count 132.0 ± 44.3, \(p < 0.01\)) compared with the control (OD1; 518.3 ± 59.1). There was a significant reduction in nocifensive behaviors for [R26A]gHwTx-IV (182.7 ± 10.0, \(p < 0.01\)), comparable with that of HwTx-IV, at the same dose (Fig. 7). There was a small but significant difference in nocifensive behavior for gHwTx-IV (308.0 ± 28.2, \(p < 0.05\)), although this peptide showed a delayed inhibitory effect during the first 10 min post-injection, with a nocifensive behavior count comparable with the control (Fig. 7). After this delay period, nocifensive behaviors were reduced to a similar level to HwTx-IV and [R26A]gHwTx-IV for the remaining 20 min of the experiment.

Discussion

Na\(_{\text{v}}\).1.7 is a promising target for the development of novel therapeutics for the treatment of pain (19, 61, 62). Spider GMTs, which are potent Na\(_{\text{v}}\) modulators, are being pursued as peptide drug leads for Na\(_{\text{v}}\).1.7 (63). Because of the proposed affinity of some GMTs for lipid bilayers surrounding NaVs (28, 33, 34, 37, 53, 64), here we have used gHwTx-IV to examine how mutations to specific GMT residues known to interact with the channels (38, 48) can also influence affinity for lipid bilayers and selectivity for Na\(_{\text{v}}\).1.7.

Point mutations to the positively-charged residues on loop 4 of gHwTx-IV reduce but do not eliminate affinity for model lipid bilayers (Fig. 4). This places [R26A]gHwTx-IV, [K27A]gHwTx-IV, and [R29A]gHwTx-IV in between gHwTx-IV and HwTx-IV with respect to affinity for neutral POPC and anionic POPC/POPS lipid bilayers. When affinity of HwTx-IV for model lipid membranes was first examined, this GMT showed no binding to lipid bilayers (34, 38). We then engineered gHwTx-IV to increase affinity for model lipid membranes by reducing the anionic contribution to the overall surface charge of the peptide and by increasing the size of the hydrophobic patch (34). At the time, we proposed that the peptide may bind to the lipid bilayer using a membrane interaction face (Fig. 8A), consisting primarily of hydrophobic residues in a manner similar to ProTx-I and ProTx-II (34, 37, 53). The observed change in affinity for lipid bilayers of the three new analogues compared with gHwTx-IV suggests that Arg-26, Lys-27, and Arg-29 also contribute to the membrane-binding face residues important for affinity for the lipid bilayers, most probably through electrostatic interactions with anionic moieties on the lipid bilayer (Fig. 8A). In a study conducted by Henriques \textit{et al.} (37), [E17K]ProTx-II showed increased affinity for POPC/POPS bilayers compared with the WT toxin. They propose that the introduction of a cationic residue increases the toxin’s affinity for the anionic phospholipid headgroups (37), which is in agreement with the results of this study. The opposite effect was observed by Nishizawa \textit{et al.} (65), who found that a mutant of GsMTx4 with increased negative charge, [K15E]GsMTx4, showed an increase in POPC/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) affinity.

[R26A]gHwTx-IV, [K27A]gHwTx-IV, and [R29A]gHwTx-IV displayed improved activity at Na\(_{\text{v}}\).1.7 compared with activity of HwTx-IV at the same channel (Fig. 5). As before, gHwTx-IV was 4-fold more potent than HwTx-IV (34). [R29A]gHwTx-IV and [K27A]gHwTx-IV displayed similar potency compared with gHwTx-IV, and [R26A]gHwTx-IV was 21-fold more potent than HwTx-IV at Na\(_{\text{v}}\).1.7 (Fig. 5 and Table 1). As mentioned, the basic residues on loop 4 of HwTx-IV have been proposed to form important interactions with anionic amino acids on the S3 and S4 loop of domain II on Na\(_{\text{v}}\)s (46, 48), as well as the anionic amino acids on the S1 and S2 loop (50, 51). In the mutagenesis study by Minassian \textit{et al.} (48), reductions in potency at Na\(_{\text{v}}\).1.7 and Na\(_{\text{v}}\).1.2 were observed for [R26A]HwTx-IV and [K27A]HwTx-IV. In the same study, [R29A]HwTx-IV maintained potency equivalent to HwTx-IV but lost potency at Na\(_{\text{v}}\).1.2 (48). Revell \textit{et al.} (24) made similar observations from their alanine scan, where activity at Na\(_{\text{v}}\).1.7 and Na\(_{\text{v}}\).1.5 was examined. The study showed that among other HwTx-IV analogues, [R26A]HwTx-IV, [K27A]HwTx-IV, and [R29A]HwTx-IV maintained minimal activity at Na\(_{\text{v}}\).1.5, but [R26A]HwTx-IV and [K27A]HwTx resulted in loss of activity at Na\(_{\text{v}}\).1.7 compared with HwTx-IV (24). Revell \textit{et al.} (24) further optimized HwTx-IV potency at Na\(_{\text{v}}\).1.7 by generating a [E1G,E4G,Y33W]HwTx-IV triple mutant, which was also investigated by Rahnama and co-workers (59) and shown to be 2–3-fold selective for Na\(_{\text{v}}\).1.7 over Na\(_{\text{v}}\).1.1, Na\(_{\text{v}}\).1.2, Na\(_{\text{v}}\).1.3, and Na\(_{\text{v}}\).1.6. Here, we have shown that

### Table 3

| Peptide      | Na\(_{\text{v}}\).1.1 | Na\(_{\text{v}}\).1.2 | Na\(_{\text{v}}\).1.3 | Na\(_{\text{v}}\).1.4 | Na\(_{\text{v}}\).1.5 | Na\(_{\text{v}}\).1.6 | Na\(_{\text{v}}\).1.7 |
|--------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| HwTx-IV      | 0.65                 | 1.06                 | >41.2                | >41.2                | >41.2                | 23.6                 | –                     |
| gHwTx-IV     | 2.73                 | 1.47                 | 82.8                 | 324                  | >136                 | 8.64                 | –                     |
| [R26A]gHwTx-IV | 9.38                | 40.8                 | 91.7                 | >392                 | >392                 | 56.3                 | –                     |
| [K27A]gHwTx-IV | 8.98                | 12.2                 | 11.2                 | 256                  | >50                  | 22.3                 | –                     |
| [R29A]gHwTx-IV | 54.1                | 13.4                 | 273                  | 309                  | >187                 | 54.8                 | –                     |
Mutations forming [K27A]gHwTx-IV and [R29A]gHwTx-IV do not negatively affect potency at NaV1.7, whereas [R26A]gHwTx-IV shows improved potency. This could be because potential losses to affinity for the channel may be compensated for by increased lipid membrane affinity. The lipid bilayer may anchor and concentrate these gHwTx-IV analogues in the lipid membrane near the channel active site. It is possible that previous studies did not see the same effect because those mutations (R26A, K27A, and R29A) were on HwTx-IV, a peptide with less affinity for the lipid bilayer compared with gHwTx-IV.

The specific interactions between the basic residues on loop 4 of HwTx-IV and the NaV1.7 voltage-sensor domain remain unclear. Multiple binding models have been proposed using different methods, but no consensus has been reached (48, 50, 51). Additionally, because the mutations that make up gHwTx-IV and its analogues may alter the position of the peptide when binding, some interactions may differ.

Although gHwTx-IV has an increased affinity for the lipid bilayer compared with HwTx-IV, gHwTx-IV did not show an improved selectivity profile compared with HwTx-IV, with a comparable lack of selectivity against NaV1.2 and NaV1.1 and a reduction of selectivity against NaV1.3 and NaV1.6 when compared with NaV1.7 (Table 3). This lack of selectivity is most probably because gHwTx-IV is anchored in the lipid bilayer in a similar fashion for all the NaV channels, which is likely to be the same across the channel sub-types particularly in this study where all channels are studied in HEK293 cells. We propose that the improved selectivity profile for the new gHwTx-IV analogues comes from weakening some electrostatic interactions with the voltage-gated ion channels, combined with having the right amount of lipid affinity to anchor the peptides, contributing to an increase in potency at NaV1.7.

We examined the in vivo efficacy of HwTx-IV, gHwTx-IV, and the most potent analogue, [R26A]gHwTx-IV, using a NaV1.7 engagement model based on intraplantar administration of the NaV1.7 agonist OD1 (52). HwTx-IV and [R26A]gHwTx-IV significantly decreased nociceptive behaviors compared with the control to a similar extent. Thus, the anti-nociceptive effects observed for [R26A]gHwTx-IV are comparable with those observed for other GMTs using the same model (21, 52, 59). Interestingly, gHwTx-IV had a delayed

Figure 6. In vitro potencies and selectivity of HwTx-IV, gHwTx-IV, and analogues. pIC50 values of all peptides at NaV1.1–1.7 expressed in HEK293 cells were determined by FLIPRTETRA membrane potential assay. Fold selectivity compared with NaV1.7 is shown. A pIC50 value of 0 was assigned to subtypes at which peptides showed no inhibitory activity up to 10 μM. Data are expressed as mean ± S.D. (n = 3).

Figure 7. Effect of intraplantar injection of 100 nM HwTx-IV, gHwTx-IV, and [R26A]gHwTx-IV in a murine model of NaV1.7-mediated nociception. A, time course of OD1-induced nociceptive behaviors over 30 min. B, pooled OD1-induced nociceptive behaviors; HwTx-IV and [R26A]gHwTx-IV each significantly reduced OD1-induced nociception (**, unpaired t test, p < 0.01). There was a significant difference in effect for gHwTx-IV, although to a lesser extent (*, unpaired t test, p < 0.05). All values expressed as mean ± S.D. (n = 3–5 animals).
inhibitory effect, showing no reduction in nocifensive behaviors over the first 10 min post-injection, then causing a decrease in nocifensive behaviors comparable with HwTx-IV over the remainder of the experiment. A similar trend was observed for m3-HwTx-IV when tested in the same OD1 pain model at 100 nM (59). This suggests that gHwTx-IV and m3-HwTx-IV, which have similar mutations, may have slower rates on 100 nM compared with HwTx-IV and [R26A]gHwTx-IV. To investigate the delayed inhibitory effect of gHwTx-IV, ρ values were measured in vitro. ρ values are concentration-dependent, and all in vivo experiments were conducted at 100 nM, so ρ values were measured in vitro both at their individual IC_{90} concentrations and at 100 nM. All three peptides showed different ρ values at their individual IC_{90} concentrations but similar values at 100 nM. At their individual IC_{90} concentrations, [R26A]gHwTx-IV had the highest ρ value, followed by gHwTx-IV, and HwTx-IV had the lowest ρ value. These results do not provide a clear explanation for the delayed in vivo activity of gHwTx-IV, as no apparent relationship exists between them. The in vitro order of potencies of the peptides was also not reflected in the in vivo experiments. Although [R26A]gHwTx-IV was 21-fold more potent than HwTx-IV, they showed similar potencies for the entire duration of the NaV1.7 engagement model, as did gHwTx-IV during the latter 20 min of the experiment.

Optimizing GMTs to access their targets in vivo remains an area for future work if these peptides are to be converted into efficacious drugs. Both HwTx-IV and the most selective analogues of ProTx-II remain toxic in animal models, resulting in death and possible histaminergic effects, respectively (43, 45). Gonçalves and co-workers (44) have recently proposed that HwTx-IV’s off-target activity at NaV,1.6 is responsible for neuromuscular toxicity (44); therefore, an area for future investigations could be to examine whether the selectivity and efficacy of [R26A]gHwTx-IV is accompanied by a loss of neuromuscular toxicity in vivo or whether systemic dosing leads to motor side effects.

Taking the in vivo and in vitro data together, we have shown here that it is possible to engineer selective modulators of NaV,1.7 while considering interactions with the lipid bilayers. The in vitro data give only a partial understanding of GMT behavior and further in vivo target engagement studies are therefore crucial to fully appreciate the biological activity of NaV,1.7 modulators.

**Conclusion**

Here, we have considered all three components of the trimolecular complex by investigating peptide–lipid bilayer as well as peptide–channel interactions in the quest to design potent and selective modulators of NaV,1.7. As seen for [R26A]gHwTx-IV, [K27A]gHwTx-IV, and [R29A]gHwTx-IV, peptides do not require maximal affinity for the lipid bilayer to display an increase in potency at NaV,1.7. Instead, for this suite of peptides, an intermediate affinity for lipid bilayers somewhere between gHwTx-IV and HwTx-IV is optimal for potency and selectivity. GMTs are probably adapted more for contact with the channel than for contact with the lipid bilayer (28); therefore, we propose that the tripartite relationship formed by gHwTx-IV and its analogues occurs such that the peptides are nestled between the extracellular loops formed by S3 and S4 and S1 and S2 and probably interact with the phospholipid headgroups in this region (Fig. 8B). We have previously discussed the possibility of this binding mode (33), and others have used molecular dynamics combined with channel mutagenesis studies on similar GMTs to demonstrate the same binding mode (48, 66, 67). X-ray crystallography has recently been used to support this GMT-binding mode, showing ProTx-II interacting with the membrane (42). Alternatively, the GMTs are first attracted to the anionic environment surrounding voltage-gated ion channels via electrostatic interactions, then concentrated in the lipid bilayer, and oriented externally near the S3 and S4-binding site on domain II of NaV,1.7 (33, 34, 37, 39–42).

In our hands, every spider GMT has shown a distinct set of behavioral patterns with respect to the lipid bilayer and voltage-gated sodium channels (28); therefore, we believe it is not possible to make sweeping statements about GMT behavior. Nevertheless, as we have shown here with HwTx-IV and previously with ProTx-II (37), detailed studies on individual GMTs can produce novel insight and approaches to optimizing these peptides for use as potential drugs for pain and other NaV-dependent pathologies. We anticipate that new technologies, including cryogenic EM, will help us to overcome current limitations and allow future studies to focus on investigating the three components (GMTs, model lipid bilayers, and voltage-gated ion channels) simultaneously (42, 68), providing a complete picture of the different interactions.

**Materials and methods**

**Peptide synthesis**

Peptides were assembled using Fmoc (N-(9-fluorenyl)me-thoxycarbonyl) chemistry on a Rink amide resin (for C-terminal amidation) on a scale of 0.25 mmol/g using an auto-
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mated Symphony peptide synthesizer (Gyros Protein Technologies Inc., Tucson, AZ), as described previously (28, 34, 69). Side-chain protecting groups for HwTx-IV, ghHwTx-IV, [R26A]gHwTx-IV, and [K27A]gHwTx-IV were Arg(Pbf), Asp(tBu), Asn(Trt), Cys (Trt), Gln(Trt), Glu(tBu), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). Additionally, as described, Acm was used for Cys II and Cys V and 4-MeBzl for Cys I and Cys IV to facilitate orthogonal oxidation of [R29A]gHwTx-IV. Cleavage from resin and removal of side-chain protecting groups (except Acm and 4-MeBzl) was simultaneously achieved in 96% (v/v) TFA, 2% (v/v) trisopropylsilane, and 2% (v/v) H₂O for 2.5 h followed by diethyl ether trituration prior to solubilizing the peptide in 45% (v/v) acetonitrile (ACN), 0.05% (v/v) TFA (solvent AB) followed by lyophilization. Reduced peptides were purified using reverse-phase HPLC (RP-HPLC) as before (28, 34, 69).

Oxidative folding of inhibitor cystine knot peptides

As described previously (24, 34, 38), oxidation of HwTx-IV was achieved in 16 h using 5 mM GSH, 0.5 mM GSSG, 0.1 M Tris (pH 8) at room temperature for 16 h (34, 38). ghHwTx-IV, [R26A]gHwTx-IV, and [K27A]gHwTx-IV were oxidized for 48 h at room temperature using 5 mM GSH, 1 mM GSSG, 0.1 M Tris, 10% (v/v) isopropyl alcohol at pH 8 (24, 34).

For [R29A]gHwTx-IV (Fig. 2), the Trt-protecting groups on Cys III and Cys VI were removed during cleavage, and the disulfide bridge between these free cysteines was formed by incubating the peptide (0.1 mg/ml) in 30% (v/v) DMSO/H₂O for 24 h. The peptide was purified using previously described semi-preparatory methods (28) and lyophilized prior to removal of Acm (or peptide injected). As described previously (34, 73), data were fitted to a one-site specific binding curve, and maximum P/L (mol/mol) was extrapolated from line-of-best-fit using GraphPad Prism, version 8.0.2 (GraphPad Software Inc, San Diego, CA).

Cell culture

HEK293 cells heterologously expressing human Naᵥ₁.1, Naᵥ₁.2, Naᵥ₁.3, Naᵥ₁.4, Naᵥ₁.5, Naᵥ₁.6, and Naᵥ₁.7 containing α and β1 subunits (SB Drug Discovery, Glasgow, Scotland, UK) were maintained at 37°C in a 5% CO₂-humidified incubator within T75 flasks containing minimal essential medium Eagle’s, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, and 1% (v/v) GlutaMAX. Selection antibiotics included 600 µg/ml gentamicin for all the cell lines, and 2 µg/ml blasticidin for Naᵥ₁.1, Naᵥ₁.3, Naᵥ₁.4, and Naᵥ₁.5. Naᵥ₁.2, Naᵥ₁.6, and Naᵥ₁.7 contained 4 µg/ml blasticidin. Naᵥ₂.4 also contained 500 µg/ml Zeocin. Replicating cells were subcultured at 70~80% confluence using 0.1% (v/v) trypLE express reagent (Thermo Fisher Scientific, Waltham, MA).

Potency and affinity determination by automated patch-clamp electrophysiology

Automated patch-clamp electrophysiology assays were performed as described previously using a QPatch-16 automated electrophysiology platform (Sophion Bioscience, Ballerup, Denmark) (74). In brief, HEK293 cells were dissociated with trypLE Express (Invitrogen) and suspended in Ham’s F-12 with 25 mM HEPES, 100 units/ml penicillin-streptomycin, and 0.04 mg/ml soybean trypsin inhibitor, and then stirred for 30~60 min. Extracellular solution contained (mM) 70 NaCl, 70 choline chloride, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose adjusted to pH 7.4, 305 mosm. For subtypes 1.1, 1.2, 1.3, and 1.6, concentrations of NaCl were increased to 140 mM to adjust for smaller currents. Intracellular solution contained (mM) 140 CsF, 1 EGTA, 5 CsSO₄, 10 HEPES, and 10 NaCl adjusted to pH 7.3 with CsOH, 320 mosm.

Peptides were diluted in extracellular solution with 0.1% BSA. Assays were run with a holding potential of −90 mV pulsed to −20 mV for 50 ms at 0.05 Hz. Activity assays were
performed with 5-min incubation periods after each peptide addition. To measure $\tau$ values, 100 nM HwTx-IV, 20 nM gHwTx-IV, and 10 nM [R26A]gHwTx-IV were added to cells and incubated for 20 min. Inhibition was quantified by measuring peak current, and all values were normalized to buffer control. Concentration–response curves were plotted with GraphPad Prism, version 8.0.2 (GraphPad Software Inc.), fitted with a four-parameter Hill equation with variable Hill coefficient. Time constants of block data were also plotted with GraphPad Prism, and $\tau$ values were calculated by fitting data to a one-phase decay. Data are presented as mean ± S.E.

**Examination of subtype selectivity using FLIPR**

The FLIPRTETRA plate reader (Molecular Devices, Sunnyvale, CA) was used to examine activity of the peptides at NaV channels as described previously (52). In brief, freshly-dissociated cells were plated into 384-well clear-bottom black-walled imaging plates (Corning, NY) at a density of 10,000–15,000 cells per well. After 48 h, the growth media were removed from the wells, and the cells were loaded with 20 µl per well red membrane potential dye (Molecular Devices) diluted in physiological salt solution (PSS; composition (mM): NaCl 140, glucose 11.5, KCl 5.9, MgCl2 1.4, Na2HPO4 1.2, NaHCO3 5, CaCl2 1.8, HEPES 10 (pH 7.4)) according to the manufacturer's instructions for 30 min at 37 °C, 5% CO2. Peptides were diluted in PSS, 0.1% (w/v) BSA and added to the cells to achieve concentrations ranging from 10 µM to 0.3 nM and incubated for 5 min. NaV channels were stimulated using 60 µM veratridine, and changes to membrane potential were measured with a cooled CCD camera (excitation 515–545 nm, emission 565–625 nm) with reads taken every 1 s for 10 s before (baseline values) and for 300 s after addition of veratridine. PSS and 0.1% (w/v) BSA was used as a negative control. ScreenWorks 3.2.0.14 (Molecular Devices) was used to compute the area under the curve over 300 s, and the data were plotted and analyzed using GraphPad Prism, version 8.0.2 (GraphPad Software Inc.), to quantify inhibitory effects of the peptides. To calculate concentration–response curves, a four-parameter Hill equation with variable Hill slope was fitted to the data. All data are expressed as the mean ± S.E. and are representative of at least three independent experiments (three wells per experiment). Potency of each peptide at off-targets is compared with potency of the specific peptide at NaV,1.7 (Table 3).

**In vivo NaV,1.7 target engagement**

Animal ethics approval was obtained from the Animal Ethics Committee of University of Queensland. All experiments were conducted in accordance with local and national regulations and the International Associations for the Study of Pain Guidelines for the Use of Animals in Research. Male C57BL/6J mice aged 6–8 weeks (20–25 g) were housed in 12-h light/dark cycles with access to food and water ad libitum. To assess the in vivo effect of peptide analogues, an OD1-induced model of NaV,1.7 target engagement was used as described previously (52).

Briefly, the NaV,1.7-selective α-scorpion toxin OD1 (300 nM) was diluted in phosphate-buffered solution and 0.1% (w/v) BSA. Under brief and light anesthesia (3% (v/v) isoflurane), mice were administered OD1 (40 µl of 300 nM) via shallow intraplantar injection into the dorsal hind paw. Animals received OD1 alone (control, n = 3) or were co-administered OD1 with gHwTx-IV or R26A[gHwTx-IV] (40 µl of 100 nM, n = 3). Following injection, mice were allowed to recover in polyvinyl boxes and were video recorded for 30 min post-injection. Spontaneous nocifensive behaviors (paw lifts, kicks, shakes, and flinches) were counted by a blinded observer.

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