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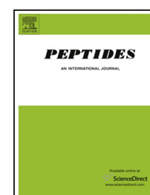

Potency optimization of Huwentoxin-IV on $hNa_v1.7$: A neurotoxin TTX-S sodium-channel antagonist from the venom of the Chinese bird-eating spider *Selenocosmia huwena*

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ABSTRACT

The spider venom peptide Huwentoxin-IV (HwTx-IV) **1** is a potent antagonist of $hNa_v1.7$ (IC_{50} determined herein as 17 ± 2 nM). $Na_v1.7$ is a voltage-gated sodium channel involved in the generation and conduction of neuropathic and nociceptive pain signals. We prepared a number of HwTx-IV analogs as part of a structure–function study into $Na_v1.7$ antagonism. The inhibitory potency of these analogs was determined by automated electrophysiology and is reported herein. In particular, the native residues Glu^1, Glu^4, Phe^6 and Tyr^{33} were revealed as important activity modulators and several peptides bearing mutations in these positions showed significantly increased potency on $hNa_v1.7$ while maintaining the original selectivity profile of the wild-type peptide **1** on $hNa_v1.5$. Peptide **47** (Gly^1, Gly^4, Trp^{33}-HwTx) demonstrated the largest potency increase on $hNa_v1.7$ (IC_{50} 0.4 ± 0.1 nM).
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1. Introduction

A long-standing inability of small molecules to fully address chronic pain has forced researchers to engage new strategies; one approach is the development of biologics targeting ion channels [2]. Ion channels allow sensory detection and transmission of electrical stimuli to the brain which may be interpreted as chronic pain in individuals where channelopathies or neuropathies exist. Hence, chronic pain may be classified as nociceptive (initiated peripherally by nociceptors), or neuropathic (resulting from nerve damage) [4]. One notable biologic success is the polypeptide Ziconotide, approved by the US Food and Drug Association in 2004 (Prialt, Azur Pharma Ltd., Azur Pharma, Philadelphia, USA) for treatment of severe chronic pain in patients who do not respond to or tolerate existing treatments [14]. Ziconotide, the synthetic form of the natural conotoxin ω-MVIIA, is a venom component of the cone snail *Conus magus* [5] which antagonizes centrally located *N*-type voltage-gated calcium channels following intrathecal injection. However, intrathecal dosing is the most expensive and invasive route of drug administration, carrying with it significant health risks, including post dose infection and risk of spinal nerve damage. For these reasons the use of Ziconotide is restricted to individuals suffering with severe chronic pain, e.g., cancer pain

Abbreviations: ACF, animal-component free; BSA, bovine serum albumin; $CaCl_2$, calcium chloride; CD, circular dichroism spectroscopy; CHO, Chinese hamster ovary; CNS, central nervous system; CO_2, carbon dioxide; CsF, cesium fluoride; CsOH, cesium hydroxide; DIC, diisopropylcarbodiimide; DMEM, Dulbecco's modified Eagle's medium; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; ESI, electrospray ionization; FBS, fetal bovine serum; GSH, reduced *L*-glutathione; GSSG, oxidized (−)-glutathione; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; KCl, potassium chloride; KOH, potassium hydroxide; RP-HPLC, reversed-phase high-performance liquid chromatography; HwTx, huwentoxin; HwTx-IV, huwentoxin-IV: a spider toxin from the venom of *Selenocosmia huwena* (also known as *Ornithoctonus huwena* and *Haplopelma schmidti*); IC_{50}, half maximal (50%) inhibitory concentration; ICK, inhibitory cystine knot; LC/MS, liquid chromatography–coupled mass spectrometry; MeCN, acetonitrile; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; NaOH, sodium hydroxide; $Na_v1.7$, voltage-gated sodium channel isoform 1.7; nm, nanometers; NMP, *N*-methylpyrrolidinone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PBS, phosphate buffered saline; PTFE, polytetrafluoroethylene; SAR, structure activity relationship; ^{t}Bu, tertiary-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, triphenylmethyl; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; TTX-S, Tetrodotoxin-sensitive; UV, ultraviolet.

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[1]. Despite its restrictions, the approval of Ziconotide has lead to renewed interest in the development of other venom peptides as therapeutics for chronic pain [12], notably antagonists of TTX-sensitive voltage-gated sodium channels such as $Na_v1.7$ [13].

$Na_v1.7$ is preferentially expressed in dorsal root ganglia and sympathetic neurons of the peripheral nervous system and is therefore accessible systemically, removing the need for intrathecal dosing. Rare congenital defects resulting in mutations to $Na_v1.7$ have given rise to several diagnostic channelopathies, implying significant therapeutic potential. Gain of function mutations of *SCN9A*, the gene encoding $Na_v1.7$ may result in neuronal hyperactivity, leading to inherited erythromelalgia (IEM) or paroxysmal extreme pain disorder (PEPD). Conversely, loss of function mutations may result in a congenital insensitivity to pain (CIP) without apparently affecting autonomic or cognitive behavior [3]. Although there have been several reports of peptide toxin blockers of $Na_v1.7$, remarkably few have exhibited selectivity profiles requisite of clinical candidates.

Huwentoxin-IV (**1**) was identified as a potential lead in the development of potent and selective antagonists of $hNa_v1.7$. HwTx-IV is a 35 amino acid neurotoxic polypeptide secreted as a venom component of the Chinese bird-eating spider *Selenocosmia huwena* and is a member of the inhibitory cystine knot (ICK) super-family of mini-proteins [8]. ICKs share a structural motif characterized by a triple β-sheet constrained by three highly conserved intramolecular disulfide bonds which collectively form a rigid structural fold [11]. Owing to their small size and high physicochemical stability the ICK motif is an excellent scaffold for drug design [9]. Herein we describe rational synthetic optimization of the inhibitory potency of HwTx-IV on $hNa_v1.7$. HwTx-IV acts selectively on tetrodotoxin-sensitive (TTX-S) voltage-gated sodium channels, preferentially inhibiting neuronal subtypes $rNa_v1.2$ ($IC_{50} \sim 150$ nM), $rNa_v1.3$ ($IC_{50} \sim 350$ nM) and $hNa_v1.7$ ($IC_{50} \sim 26$ nM) over the muscle subtypes $rNa_v1.4$ and $hNa_v1.5$ ($IC_{50} > 10$ μM) [17–20]. Although HwTx-IV inhibits centrally expressed $rNa_v1.2$ with considerable potency, effects of its inhibition can likely be avoided by systemic dosing to access the peripheral Na_v isoforms only. HwTx-IV inhibits to a far lesser extent $Na_v1.5$, expressed in cardiac tissue, hence, its potency and selectivity profile suggests considerable potential for development as a treatment for neuropathic and nociceptive pain. Since $Na_v1.7$ is present in neither cardiac muscle nor CNS neurons, blockers of $Na_v1.7$ are expected to exhibit fewer side-effects than current pain treatments. In the present study, a series of synthetic HwTx-IV analogs were prepared and profiled for sodium ion channel activity on both $hNa_v1.7$ and $hNa_v1.5$ cell lines using automated electrophysiology.

2. Experimental

2.1. Peptide synthesis

2.1.1. Materials

N-α-Fmoc-L-amino acids were obtained from Bachem AG, Switzerland. NovaSyn® TGR (TentaGel Rink) synthesis resin was obtained from Novabiochem, Merck Biosciences, Darmstadt, Germany. All peptides were prepared by automated microwave-assisted synthesis (CEM Liberty) using the Fmoc/^{t}Bu protocol. Cysteine (Cys), asparagine (Asn) and glutamine (Gln) were incorporated as their sidechain trityl (Trt) derivatives. Tryptophan (Trp) and lysine (Lys) were incorporated as their sidechain Boc derivatives. Serine (Ser), threonine (Thr) and tyrosine (Tyr) were incorporated as sidechain ^{t}Bu ethers, and aspartate (Asp) and glutamate (Glu) as their sidechain O^{t}Bu esters. Finally, arginine (Arg) was incorporated as the sidechain Pbf derivative. Synthesis reagents were obtained from Sigma–Aldrich, Dorset, United Kingdom. Solvents were obtained from Merck, Darmstadt, Germany at the highest grade available and used without further purification.

2.1.2. Synthesis of linear peptides

Unless otherwise stated, all peptides were prepared as C-terminal carboxamides on NovaSyn® TGR resin (initial substitution 0.24 mmol/g). All amino acids were coupled for 15 min each at 70 °C, however, Fmoc-Cys(Trt)-OH was coupled twice at 50 °C for 15 min. Syntheses were carried out on a 0.12 mmol scale (500 mg synthesis resin) and the instrument was set to deliver 1 mmol (∼8 equiv.) of each of the following reagents: *N*-α-Fmoc-L-amino acid (2.5 M solution in NMP), HOBt (0.4 M solution in DMF) and DIC (0.4 M solution in NMP). Residual functionality was capped using a solution of acetic anhydride (0.5 M) and pyridine (0.5 M) in DMF for 5 min at 60 °C following each acylation cycle.

2.1.3. Cleavage and purification of linear peptides

Crude linear peptides were cleaved from the resin by treatment with a cocktail consisting of TFA (87.5%), TIPS (2.5%), water (2.5%), liquefied phenol (2.5%), thioanisole (2.5%) and EDT (2.5%, v/v) for 3× 1 h at room temperature with agitation. Cleavage aliquots were combined, concentrated by rotary evaporation and precipitated by addition of 10 volumes of cold diethyl ether, isolating the solids by centrifugation at 3500 rpm. Crude peptides were dried under a flow of dry nitrogen, reconstituted in 20% MeCN/water (v/v) and filtered (0.4 μm, PTFE) prior to purification by RP-HLPC. Crude peptides were chromatographed using an Agilent Polaris C8-A stationary phase (21.2 mm × 250 mm, 5 μm) eluting at 20 mL min^{-1} with a linear solvent gradient of 5–50% MeCN (0.1% TFA, v/v) in water (0.1% TFA, v/v) over 30 min using a Varian SD-1 Prep Star binary pump system, monitoring by UV absorption at 210 nm. The desired pooled fractions containing the linear reduced peptide were concentrated and lyophilized prior to folding.

2.1.4. Oxidative folding of linear peptides

Reduced linear peptides were reconstituted in 1:1 MeCN/water (∼10 mg/mL) and diluted to a final concentration of approximately 0.1 mg/mL in freshly de-oxygenated folding buffer consisting of 0.1 M Tris–HCl (pH 8.0), 10% (v/v) propan-2-ol, 5.0 mM in reduced glutathione (GSH) and 1.0 mM in oxidized glutathione (GSSG). The crude folding mixture was filtered (0.4 μm, PTFE) and re-adjusted to pH 8.0 by dropwise addition of 1 M NaOH (aq) or 1 M TFA (aq) with stirring. Re-naturation was monitored by analytical RP-HPLC over the course of approximately 48 h at room temperature. When the crude folding mixture was shown to contain a new well defined peptide species and judged by RP-HPLC to be complete the reaction was quenched by acidification with MeCN/water/TFA (1:1:1) to an end point of pH 2.0, and the desired oxidized peptide was isolated by RP-HPLC as described below.

2.1.5. Purification of folded peptides

Crude folded peptides were purified to homogeneity by direct application of the folding solution to an Agilent Polaris C8-A stationary phase (21.2 mm × 250 mm, 5 μM) eluting at 20 mL min^{-1} with a linear gradient of 5% to 50% MeCN (0.1% TFA, v/v) in water (0.1% TFA, v/v) over 30 min on a Varian SD-1 Prep Star binary pump system, monitoring by UV absorption at 210 nm. The desired pooled fractions were concentrated and lyophilized prior to storage at −20 °C.

2.1.6. Peptide analysis and characterization

Purified linear (cysteine) and purified folded (cystine) peptides were characterized by single quadrupolar LC/MS using a Waters Mass Lynx 3100 platform. Analytes were chromatographed by elution on a Waters X-Bridge C18 stationary phase (4.6 mm × 100 mm, 3 μM) using a linear binary gradient of 10–90% MeCN (0.1% TFA,

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