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PII: S0006-2952(18)30364-2
DOI: <https://doi.org/10.1016/j.bcp.2018.08.038>
Reference: BCP 13271

To appear in: *Biochemical Pharmacology*

Received Date: 29 May 2018
Accepted Date: 22 August 2018

Please cite this article as: L. Ma, Y.K.Y. Chin, Z. Dekan, V. Herzig, C. Yuen Chow, J. Heighway, S. Wing Lam, G.J. Guillemain, P.F. Alewood, G.F. King, Novel venom-derived inhibitors of the human EAG channel, a putative antiepileptic drug target, *Biochemical Pharmacology* (2018), doi: <https://doi.org/10.1016/j.bcp.2018.08.038>

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**Novel venom-derived inhibitors of the human EAG channel,
a putative antiepileptic drug target**

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Category: Neuropharmacology

Key words: Kv10.1, hEAG1, hERG, ion channel, epilepsy

ABSTRACT

Recently, we and other groups revealed that gain-of-function mutations in the human ether à go-go voltage-gated potassium channel hEAG1 (K_v10.1) lead to developmental disorders with associated infantile-onset epilepsy. However, the physiological role of hEAG1 in the central nervous system remains elusive. Potent and selective antagonists of hEAG1 are therefore much sought after, both as pharmacological tools for studying the (patho)physiological functions of this enigmatic channel and as potential leads for development of anti-epileptic drugs. Since animal venoms are a rich source of potent ion channel modifiers that have been finely tuned by millions of year of evolution, we screened 108 arachnid venoms for hEAG1 inhibitors using electrophysiology. Two hit peptides (Aa1a and Ap1a) were isolated, sequenced, and chemically synthesised for structure-function studies. Both of these hEAG1 inhibitors are C-terminally amidated peptides containing an inhibitor cystine knot motif, which provides them with exceptional stability in both plasma and cerebrospinal fluid. Aa1a and Ap1a are the most potent peptidic inhibitors of hEAG1 reported to date, and they present a novel mode of action by targeting both the activation and inactivation gating of the channel. These peptides should be useful pharmacological tools for probing hEAG1 function as well as informative leads for the development of novel anti-epileptic drugs.

1. Introduction

Voltage-gated potassium (K_v) channels promote K^+ efflux upon membrane depolarization in excitable cells, thereby helping to restore the resting membrane potential. The human ether *à* go-go channel (hEAG1, also known as $K_v10.1$ and KCNH1), encoded by the *KCNH1* gene, is a member of the K_v channel family. Although EAG1 channels contain several intracellular domains, they adopt a classical homotetrameric K_v channel architecture, with each of the four subunits containing six membrane-spanning α -helices (S1–S6). Helices S1–S4 serve as voltage sensor units, while the S5–S6 helices from each subunit come together to form the channel pore [1, 2].

EAG1 mediates rapid, potassium-selective, non-inactivating outward currents upon membrane depolarization [2, 3]. The channel is preferentially expressed in the central nervous system (CNS), with high abundance in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus and cerebellum [4]. However, the neurophysiological role of EAG1 remains elusive, in part due to the lack of selective inhibitors [2]. Recently, *de novo* heterozygous *KCNH1* mutations were reported in patients with developmental disorders and infantile-onset epilepsy, including Temple–Baraitser syndrome [5] and Zimmermann–Laband syndrome [6].

Under normal circumstances, expression of *KCNH1* is barely detectable outside the CNS, but the channel is robustly overexpressed in a variety of human tumours of variable tissue origin, including liver, prostate, ovary, colon, thyroid, skin and bone cancers [7-9]. Moreover, overexpression of *KCNH1* correlates with poor prognosis for sarcoma, colon cancer, ovary carcinoma and acute myeloid leukemia [10]. Subcutaneous injection of *KCNH1*-transfected cells into immunodeficient mice results in invasive tumor development and progression [11].

Given the important role of hEAG1 in neurodevelopment and tumorigenesis, hEAG1 inhibitors might have therapeutic potential. Unfortunately, currently available blockers of hEAG1, such as imipramine and astemizole, have either low potency or poor specificity, particularly over the closely related hERG ($K_v11.1$) channel [12]. The venoms of spiders and other venomous invertebrates evolved to target the nervous system of prey and predators and consequently they are highly enriched with ion channel modifiers [13]. Indeed, a peptidic inhibitor of hEAG1 was recently isolated from sea anemone venom [14]. Here we used two-electrode voltage-clamp (TEVC) electrophysiology to screen a panel of arachnid venoms for inhibitors of hEAG1. We identified two disulfide-rich spider-venom peptides that potently inhibit hEAG1. Both peptides contain an inhibitor cystine knot (ICK) motif [15] and are highly stable in biological fluids. In contrast to most venom-derived K_v channel inhibitors, these peptides are gating modifiers rather than pore blockers. One of these peptides (Ap1a) is the first reported hEAG1 inhibitor that has

both high potency (IC_{50} 236 nM) and >30-fold selectivity over hERG, and hence it should be a useful pharmacological tool for studying hEAG1 function.

2. Methods and Materials

2.1 Chemicals

All chemicals used in this study were from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise stated.

2.2 Screen of arachnid venoms against hEAG1 using TEVC electrophysiology

An in-house collection of 108 arachnid venoms (63 spider and 45 scorpion venoms, Table 1) was screened for activity against hEAG1. Spider venoms were obtained by electrostimulation, whereas scorpion venoms were mainly obtained by aggravation, sometimes assisted by additional electrostimulation. Venom was collected, lyophilised and stored at -80°C until required. Venoms and venom fractions were assessed against hEAG1 heterologously expressed in *Xenopus* oocytes. Briefly, the hKCNH1-pcDNA3.1 plasmid [5] was linearized, then capped cRNAs were synthesized using a T7 mMESSAGE-mMACHINE transcription kit (Thermo Fisher Scientific Australia, Scoresby VIC, Australia). Stage V-VI oocytes were harvested from ovarian lobes of anaesthetised *Xenopus laevis* frogs (University of Queensland Animal Ethics Approval QBI/AIBN/087/16) and defolliculated by treatment with collagenase type I. Oocytes injected with 20 ng of hKCNH1 cRNA were incubated for 24 h at 17°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl_2 , 2 mM MgCl_2 , 5 mM HEPES, pH 7.4), supplemented with 2.5 mM sodium pyruvate, 50 $\mu\text{g}/\text{ml}$ gentamicin and 2.5% horse serum (Thermo Fisher Scientific) prior to electrophysiological recordings. TEVC recordings were made at room temperature (20 – 22°C) using an Axoclamp 900A amplifier (Molecular Devices, San Jose, CA, USA). Standard borosilicate microelectrodes used for recording had resistance of 0.5 – $1\text{ M}\Omega$ when filled with 3 M KCl solution. Stimulation, data acquisition, and analysis were performed using pCLAMP 10 software (Molecular Devices). Oocytes were clamped at -120 mV and outward currents elicited by a 50-ms depolarising steps to $+80\text{ mV}$ every 10 s.

2.3 Isolation of hEAG1 inhibitors from spider venoms

Lyophilised venom (1 mg) was diluted with 5% solvent B (90% acetonitrile (ACN), 0.05% trifluoroacetic acid (TFA) in H_2O) and loaded onto an analytical C18 reversed-phase (RP) HPLC column (Aeris Widespore 3.6 μm ; Phenomenex Australia, Lane Cove, NSW, Australia) attached to a Prominence HPLC system (Shimadzu Scientific Instruments, Rydalmere, NSW, Australia). Components were eluted at 0.7 ml/min with solvent A (99.95% H_2O , 0.05% TFA) and solvent B

using isocratic elution at 5% solvent B for 5 min, followed by a gradient of 5–20% solvent B over 5 min, then 20–40% solvent B over 40 min, and 40–80% solvent B over 5 min. hEAG1-active fractions were further fractionated using a Hypersil C18 column (150 × 4.6 mm; Thermo Fisher Scientific Australia) or a Pursuit 3 Diphenyl column (150 × 3.0 mm, 3 µm, 200 Å pore size; Agilent Technologies Australia, Mulgrave, VIC, Australia). Absorbance was measured at 214 and 280 nm using a Shimadzu Prominence SPD-20A detector.

2.4 Sequencing of peptidic hEAG1 inhibitors

Peptide masses were determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Model 4700 Proteomics Analyser (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). α -Cyano-4-hydroxycinnamic acid (CHCA) was used as matrix. RP-HPLC fractions were mixed 1:1 (v/v) with CHCA (7.5 mg/ml in 1:1 ACN/H₂O, 0.1% TFA) on a MALDI plate. MALDI-TOF spectra were collected in reflector positive mode, and reported masses are monoisotopic $M+H^+$ ions. N-terminal Edman sequencing was performed by the Australian Proteome Analysis Facility and this data was complemented by MS analysis of carboxypeptidase Y-digested peptides to obtain complete peptide sequences. Briefly, reduced and alkylated peptides were dissolved in 100 mM ammonium acetate (pH 5.5) and digested with 2 ng/µl sequencing-grade carboxypeptidase Y. The digestion at 37°C was stopped at 1, 2, 5, 10, 15, 30 or 60 min by addition of formic acid (FA) to a final concentration of 0.1%. C-terminal sequences were resolved by MS analysis of the digestion products.

2.5 Chemical synthesis of hEAG1 inhibitors

hEAG1-active peptides were assembled on a Symphony automated peptide synthesiser (Protein Technologies, Manchester, UK) on Fmoc-Rink amide (loading 0.80 mmol/g) polystyrene resin at 0.1 mmol scale. Fmoc deprotection was achieved using 30% piperidine/*N,N*-dimethylformamide (DMF) (1 × 1.5 min, then 1 × 3 min). Couplings were performed in DMF using five equivalents of Fmoc-amino acid/*O*-(1*H*-6-chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HCTU)/*N,N*-diisopropylethylamine (DIEA) (1:1:1) relative to resin loading (1 × 4 min, then 1 × 8 min). Amino acid side-chains were protected as follows: Asp(OtBu), Arg(Pbf), Cys(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu) and Trp(Boc). Cleavage from the resin and removal of side-chain protection was achieved by treatment with 94% TFA/3% triisopropylsilane (TIPS)/3% H₂O at room temperature for 2 h. After most of the cleavage solution was evaporated under a stream of N₂, the products were precipitated and washed with cold diethyl ether and lyophilised from 50% ACN/0.1% TFA/H₂O. The crude products were purified by preparative HPLC.

ESI-MS (m/z): Ap1a calc. (avg) 1410.6 $[M+3H]^{3+}$, found 1410.4; Aa1a calc. (avg) 1386.6 $[M+3H]^{3+}$, found 1386.5.

Solvents for RP-HPLC consisted of 0.05% TFA/H₂O (solvent C) and 90% ACN/0.043% TFA/H₂O (solvent D). Analytical RP-HPLC was performed on a Shimadzu LC20AT system using a Hypersil GOLD 2.1 × 100 mm C18 column (Thermo Fisher Scientific Australia) heated to 40 °C with flow rate 0.3 ml/min. A gradient of 10–55% solvent D over 30 min was used, with detection at 214 nm. Preparative RP-HPLC was performed on a Vydac 218TP1022 column (Grace, Columbia, MD, USA) using a flow rate of 16 ml/min and a gradient of 5–45% solvent D over 40 min. MS was performed on an API150 mass spectrometer (AB SCIEX Australia, Mulgrave, VIC, Australia) in positive ion mode.

Oxidative folding of peptides was achieved by mixing purified reduced peptide (1 equiv), reduced glutathione (100 equiv.) and oxidised glutathione (10 equiv.) in 6 M GnHCl (1 ml/mg peptide); this mixture was then added to a solution of 1.55 M GnHCl/0.37 M NH₄OAc (pH 8.0, 9 × volume of peptide solution) and stirred at 4°C with exposure to air for 2 days. The single major product was isolated by preparative RP-HPLC. ESI-MS (m/z): Ap1a calc. (avg) 1408.6 $[M+3H]^{3+}$, found 1408.5; Aa1a calc. (avg) 1384.6 $[M+3H]^{3+}$, found 1384.5.

2.6 Cell culture

CHO-K1 cells stably expressing hEAG1 (hEAG1-CHO cells, generously donated by Prof. Steve Petrou, The University of Melbourne, Australia) were cultured in F-12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 µg/ml hygromycin. HEK293 cells stably expressing the human voltage-gated sodium (Na_v) channel hNa_v1.2, hNa_v1.5 or hNa_v1.7 channels along with the β1 subunit (SB Drug Discovery, Glasgow, UK) were cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and variable concentrations of blasticidin and geneticin, according to the manufacturer's instructions. Cells were maintained at 37°C in a humidified 5% CO₂ incubator and they tested negative for mycoplasma. HEK293- and CHO-based cells have doubling times of about 32 h and 15 h, respectively, under the above culture conditions. Cells used for patch-clamp electrophysiology were of passage number 10–20.

2.7 Patch-clamp electrophysiology

Patch-clamp experiments were conducted at room temperature in the whole-cell configuration using an Axon MultiClamp 700B amplifier (Molecular Devices) or a QPatch 16X automated

platform (Sophion Bioscience, Ballerup, Denmark). Cells were continuously superfused with bath solution containing 140 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 8 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass and typically had a resistance of 2–5 MΩ when filled with an internal solution containing 70 mM KF, 60 mM KCl, 15 mM NaCl, 5 mM EGTA-Na and 5 mM HEPES, adjusted to pH 7.2 with NaOH. The same bath solution and internal solution were used for all patch-clamp recordings. Series resistance did not exceed 5 MΩ and was 70–80% compensated. Acquired currents were filtered at 1 kHz, sampled at 10 kHz, collected and analysed using Clampex 10.0 software (Molecular Devices) or QPatch Assay software 5.2 (Sophion Bioscience).

To quantify the inhibitory potency of peptides, hEAG1/hERG currents elicited by a depolarisation to +80 mV for 240 ms were recorded with increasing concentrations of peptide. For Na_v channels, currents were triggered at 0 mV for 50 ms. Concentration-response relationships were fitted with the Hill equation:

$$\frac{I_x}{I_{\max}} = \frac{[x]^n}{IC_{50}^n + [x]^n}$$

where I_x is the steady-state hEAG1/hERG current or peak Na_v channel current in the presence of peptide at concentration $[x]$, $I_{\max}$ is the current in the absence of peptide, IC_{50} is the peptide concentration at which inhibition was half-maximal, and n is the slope. All values are given as mean ± standard deviation (SD) for the number of biologically independent measurements indicated (n). Statistical significance was determined using a student's t -test.

To test the voltage-dependent activation of hEAG1 channels, 1-s pulses from –100 mV to +120 mV in 10-mV steps were used to activate the channel from a holding potential of –120 mV, followed by a 400-ms step to –100 mV. Currents elicited by the test pulse were normalized to $I_{\max}$, plotted against the test voltage, and fitted to a single-Boltzmann function:

$$\frac{I}{I_{\max}} = \frac{1}{1 + e^{-\frac{zF}{RT}(V - V_{\text{half}})}}$$

where $I/I_{\max}$ is the normalized current, z is the equivalent gating charge, V_{half} is the half-activation voltage, and F is Faraday's constant.

Deactivation of hEAG1 was studied by first activating the channel at +80 mV for 800 ms and then

deactivating the channel at -140 mV for 500 ms before returning to a holding potential of -120 mV. The kinetics of deactivation were analyzed by fitting the deactivation sweep at -140 mV with a double-exponential equation; τ values were compared using an unpaired t -test with Welch's correction. Steady-state inactivation was measured using a three-step protocol: starting from a holding potential of -120 mV, pulses were given from -130 mV to $+80$ mV in 15-mV steps, and then the level of inactivation was tested by an activating step at $+40$ mV.

2.8 Structure determination

NMR spectroscopy was used to determine the 3D structure of Aa1a. A 300 μ l sample of Aa1a (1 mM in 20 mM sodium phosphate, pH 7.4, 5% D_2O) was placed in a susceptibility-matched microtube (Sigma-Aldrich), and spectra acquired at $25^\circ C$ on an Avance 600 MHz spectrometer (Bruker BioSpin, Billerica, MA, USA). Resonance assignments were obtained using a combination of 2D 1H - 1H TOCSY and natural abundance 2D 1H - ^{15}N and 1H - ^{13}C HSQC spectra. Interproton distance restraints were obtained from a 2D 1H - 1H NOESY spectrum acquired with a mixing time of 250 ms. Spectra were processed using Topspin (v3.2, Bruker) and analysed using CcpNmr Analysis v2.4.1 [16]. Chemical shifts, including assignments for 99.5% of protons, were deposited in BioMagResBank under accession number 30318. TALOS-N [17] was used to derive dihedral-angle restraints and the restraint range used in structure calculations was set to twice the estimated standard deviation. The 1H - 1H NOESY spectrum was manually peak-picked, then the torsion angle dynamic package CYANA v3.97 [18] was used to automatically assign the peak list, extract distance restraints, and calculate an ensemble of structures. CYANA assigned 92.9% of NOESY cross-peaks. 200 structures were calculated based on 523 inter-proton distance restraints, 9 disulfide-bond distance restraints, and 63 dihedral-angle restraints (Table 2). The 30 structures with lowest penalty function values were analysed using MolProbity [19], and the 20 structures with highest stereochemical quality were used to represent the structure of Aa1a. The ensemble of structures is available from the Protein Data Bank (accession code 5WLX).

2.9 Biological stability of peptides

The stability of Aa1a and Ap1a was tested by adding the peptides to human cerebrospinal fluid (CSF) or human plasma at a final concentration of 2 μ M and incubating the mixture at $37^\circ C$ for periods up to 72 h (University of Queensland Human Research Ethics Approval 20160008176). De-identified CSF samples were obtained from patients undergoing lumbar puncture at St. Vincent's Hospital, Sydney, NSW, Australia (Ethics Approval SVH15/024) while male AB plasma was from Sigma-Aldrich (Catalog No. H4522). Triplicate samples were collected at each time point, and

reaction mixtures precipitated with 5 μ l of 5% TFA. For each time point, a 0.1 nmol sample was processed by LC/MS using a Kinetex C18 column (150 mm $\times$ 2.1 mm, particle size 2.6 μ m, 100 Å pore size; Phenomenex) at a flow rate of 0.2 ml/min and a gradient of 2–40% solvent B (90% ACN, 0.1% FA) in solvent A (0.1% FA) over 14 min coupled with an AB SCIEX 5600 Triple time-of-flight (TOF) mass spectrometer (cycle time 0.2751 s). Peptide peak areas were measured at triple-, quadruple- and quintuple charge states, and analysed using PeakView and MultiQuant (Applied Biosystems/Thermo Fisher Scientific). Human atrial natriuretic peptide (1 μ M; GenScript, Piscataway, NJ, USA) and the analgesic drug Prialit (i.e., ω -conotoxin MVIIA, 1 μ M; Alomone Labs, Jerusalem, Israel) were used as controls.

3. Results

3.1 A small number of arachnid venoms contain inhibitors of hEAG1

We used TEVC electrophysiology to screen arachnid venoms for inhibition of hEAG1. Heterologous expression of hEAG1 in *Xenopus* oocytes generated robust and non-inactivating outward K^+ currents upon membrane depolarization (Fig. 1). Only 9 of the 108 venoms tested (Table 1) inhibited these currents by >50%, and all of these were spider venoms. However, 21 of the 63 spider venoms (33%) inhibited hEAG1 to some extent (Table 1), which is similar to the proportion of spider venoms (40%) previously reported to contain inhibitors of $Na_v1.7$ [20].

Venoms from the tarantulas *Avicularia aurantiaca* and *A. purpurea* (Fig. 1A) were notable for significant inhibition of hEAG1 (Fig. 1B). These venoms were fractionated using RP-HPLC (Fig. 1C), then each fraction tested against hEAG1 using the same TEVC protocol. This led to isolation of two “hit” peptides with monoisotopic masses of 4148.217 Da and 4220.077 Da as measured using MALDI-TOF MS (Fig. 1D and E). Each purified peptide robustly inhibited hEAG1 (Fig. 1F). Based on the rational nomenclature proposed for venom peptides [21], these peptides were named κ -theraphotoxin-Aa1a (hereafter Aa1a) and μ/κ -theraphotoxin-Ap1a (hereafter Ap1a); the κ prefix denotes inhibition of K_v channels, the μ prefix denotes inhibition of Na_v channels (see below), ‘theraphotoxin’ indicates the taxonomic family to which these spiders belong, while the genus and species are indicated by uppercase and lowercase letters (Aa and Ap, respectively).

3.2 Primary structure of Aa1a and Ap1a

The amino acid sequences of Aa1a and Ap1a were elucidated by combining data from MS, Edman degradation, and amino acid analysis. Edman sequencing revealed the identity of the N-terminal 35 residues of Aa1a and Ap1a, while MS analysis of peptide digested briefly with carboxypeptidase Y indicated that the last amino acid residue is Phe for Ap1a and either Leu or Ile

for Aa1a, with both peptides having an amidated C-terminus. Amino acid analysis revealed that the C-terminal residue in Aa1a is Ile not Leu. Thus, as shown in Fig. 2A, both peptides consist of 36 residues, with six cysteines and amidated C-termini. The predicted mass of Aa1a and Ap1a based on these sequences (4148.81 Da and 4220.69 Da respectively) matches well with the measured mass of the purified native peptides (4148.22 Da and 4220.08 Da respectively). Aa1a and Ap1a are clearly paralogues, with 81% identity, but a BLAST search of these sequences against known spider toxins in the ArachnoServer database (www.arachnoserver.org; [22, 23] revealed none with identity >60%, suggesting that these peptides are novel. The peptide with highest homology to Aa1a and Ap1a (58–59% identity) is U₁-theraphotoxin-Gr1b, a toxin with unknown molecular target from the Chilean rose tarantula *Grammostola rosea* [24].

3.3 Three-dimensional structures of Aa1a and Ap1a

The C-terminally amidated Aa1a and Ap1a peptides were produced by solid-phase peptide synthesis and oxidatively folded in a redox buffer. The synthetic peptides co-eluted on RP-HPLC with native peptide isolated from crude venom (Fig. 2B), indicating that they adopt the native disulfide-bond architecture. The 3D structure of Aa1a was determined using NMR spectroscopy (Fig. 2C). The ensemble of 20 structures is highly precise, with an rmsd of 0.25 ± 0.08 over the backbone atoms of residues 3–34, which excludes the flexible N- and C-termini. The ensemble of structures has high stereochemical quality, with mean MolProbity score of 1.7 ± 0.1 (Table 2). The disulfide-bond connectivity was unequivocally determined from preliminary structure calculations performed without disulfide-bond restraints; the inter cystine NOEs in the NOESY spectrum [25] are consistent with the pattern Cys³-Cys¹⁸, Cys¹⁰-Cys²³ and Cys¹⁷-Cys³⁰. The 3D structure of Aa1a indicates that the three disulfides form a cystine knot in which the Cys³-Cys¹⁸ and Cys¹⁰-Cys²³ disulfide bonds and the intervening section of the polypeptide backbone form a closed loop that is pierced by the Cys¹⁷-Cys³⁰ disulfide. The overall peptide fold conforms to the ICK motif commonly adopted by spider toxins [13, 15, 26], with two of the disulfides supporting a canonical β -hairpin (Fig. 2C). The structure of Ap1a, which differs by only seven residues from Aa1a, is likely to be very similar.

Interestingly, most of the hydrophobic residues in Aa1a (F6, L7, W9, W29, W32) are stacked like the rungs of a ladder on one face of the peptide (Fig. 2D). These residues are largely conserved in Ap1a, where the equivalent residues are F6, W7, W9, W29, and W32 (see alignment in Fig. 2A). Hydrophobic patches are often found on the surface of gating-modifier spider toxins where they can engage in interactions with both the target ion channel and the lipid membrane [27].

3.4 Aa1a and Ap1a are potent inhibitors of hEAG1

Ap1a inhibited hEAG1 expressed in CHO cells with an IC_{50} of 236 ± 21 nM and Hill coefficient of 1.32 ± 0.13 (Fig. 3B,C; $n = 7$) as tested by patch-clamp electrophysiology, making it 9-fold more potent than imipramine (IC_{50} 1,800 nM; Table 3), the most commonly used hEAG1 inhibitor. Ap1a is nearly equipotent with astemizole (IC_{50} 196 nM; Table 3), the most potent known inhibitor of hEAG1 [12]. Interestingly, Ap1a does not completely inhibit channel function, with maximum inhibition of ~80% at saturating concentrations (Fig. 3C). In contrast, Aa1a is 3-fold less potent than Ap1a (IC_{50} 637 ± 59 nM, Hill coefficient 1.03 ± 0.09 ; $n = 5$), but at high concentration it completely inhibits hEAG1 (Fig. 3A and C).

From raw current traces it is clear that Aa1a and Ap1a have fast on-rates at all concentrations tested (Fig. 3A and B). However, they have significantly different off-rates. Whereas Aa1a is easily washed off (Fig. 3D), with a calculated off-rate of 19.7 ± 4.2 s ($n = 4$), the inhibitory effect of Ap1a is much less reversible (Fig. 3E), which might partly explain its higher potency. The concentration-response curves for both peptides have a Hill coefficient of ~1.0 (Fig. 3C), suggestive of a single binding site.

3.5 Aa1a and Ap1a are gating-modifiers of hEAG1

We studied the mechanism of action of Aa1a and Ap1a via patch-clamp of hEAG1-CHO cells. Activation of hEAG1 resulted in robust voltage-dependent outward K^+ currents with time-dependent slow inactivation at very positive activating potentials (Fig. 4A, left panel). Fitting the activation current-voltage (I-V) relationship data with the Boltzmann equation revealed a half-maximal activation voltage ($V_{1/2}$) of 20.8 ± 4.5 mV ($n = 14$). At concentrations corresponding to their IC_{50} values (i.e., 600 nM Aa1a, 200 nM Ap1a), both peptides caused a significant depolarising shift in the I-V relationship for channel activation: $V_{1/2}$ was shifted to 39.2 ± 4.7 mV ($n = 7$) and 37.7 ± 3.1 mV ($n = 9$), in the presence of Aa1a and Ap1a, respectively ($P < 0.0001$; Fig. 4A, right panel). As a consequence, both peptides significantly reduced channel open probability over the physiological membrane potential range. Interestingly, Ap1a, but not Aa1a, promoted voltage- and time-dependent slow inactivation of hEAG1 at open state (Fig. 4A, right).

Voltage-gated channels can also be inhibited by enhancing their deactivation. To test this possibility, we compared the deactivation of hEAG1 in the presence and absence of 600 nM Aa1a or 200 nM Ap1a. As shown in Fig. 4B, the time constant for hEAG1 deactivation (2.4 ± 0.6 ms, $n = 6$) was not significantly different from that in the presence of 600 nM Aa1a (3.8 ± 0.8 ms, $n = 6$) or 200 nM Ap1a (3.0 ± 0.7 ms, $n = 6$). Therefore, neither peptide induced a significant change in

deactivation kinetics, indicating that Aa1a and Ap1a do not inhibit hEAG1 by promoting channel deactivation.

A characteristic biophysical feature of hEAG1 is that channel activation is dramatically impacted by the pre-conditioning membrane potential. Briefly, hEAG1 activation is delayed and slower when currents are elicited from more hyperpolarised holding potentials. This phenomenon, which is commonly known as the Cole-Moore shift [28], even though this designation is not entirely appropriate [2], has been traced to charge movement between early closed states of the channel [29, 30]. Interestingly, both peptides significantly enhanced the Cole-Moore shift of hEAG1 (Fig. 4C, bottom panels). This markedly contrasts with the effect of partial deletion of the EAG domain, which almost completely eliminates the dependence of activation kinetics on the holding potential (i.e., markedly reduces the Cole-Moore shift; [31]).

With a pre-conditioning membrane potential of -120 mV, the activation kinetics at $+40$ mV was dramatically slowed down by both Aa1a (600 nM) and Ap1a (200 nM); the activation time constant (τ) increased from 0.07 ± 0.01 ms ($n = 5$) in the absence of peptides to 1.69 ± 0.37 ms ($n = 4$) and 1.29 ± 0.18 ms ($n = 6$) in the presence of Aa1a and Ap1a, respectively (Fig. 4D left). Aa1a and Ap1a also shifted the voltage dependence of the initial activation at $+40$ mV after a long pre-conditioning step in the depolarising direction, changing $V_{1/2}$ from -82.1 ± 3.2 mV ($n = 5$) to -68.0 ± 8.2 mV ($n = 6$) and -61.2 ± 8.7 mV ($n = 3$) respectively (Fig. 4D, middle). Thus, in the presence of the peptides, hEAG1 is harder to activate after being exposed to certain pre-conditioning membrane potentials within the physiological range. On the other hand, the voltage dependence of steady-state inactivation, as measured from the plateaued currents at $+40$ mV, was largely eradicated by the peptides, particularly Aa1a (Figure 4D, right). Aa1a (600 nM) shifted the effective $V_{1/2}$ of steady-state inactivation from -69.8 ± 5.9 mV ($n = 4$) to 20.5 ± 2.7 mV ($n = 3$). In contrast, 200 nM Ap1a reduced steady-state inactivation magnitude by $\sim 50\%$ without significantly changing its voltage dependence ($V_{1/2} = -63.0 \pm 5.7$ mV, $n = 3$), further demonstrating the functional difference between these peptides despite the high level of sequence identity.

3.6 Aa1a and Ap1a are selective for hEAG1 over hERG

hERG and hEAG1 are highly homologous members of the same subfamily of EAG-like K_v channels. hERG is instrumental in repolarisation of the cardiac action potential and consequently inhibition of this channel can lead to long QT syndrome and enhanced risk of sudden death [32]. For this reason, the FDA mandates that ion channel drug candidates be tested to ensure that they are not potent inhibitors of hERG.

Thus, to further examine the potential of Ap1a and Aa1a as pharmacological tools for probing hEAG1 function, we tested their ability to modulate hERG (Fig. 5A,B). As judged by IC_{50} levels, Aa1a and Ap1a were 13 and 35 times more potent on hEAG1 than on hERG1, respectively (Fig. 5C). In striking contrast, although astemizole is a good inhibitor of hEAG1 (IC_{50} 196 nM), it is not a useful pharmacological tool for selectively probing hEAG1 function because it inhibits hERG with >200-fold higher potency (IC_{50} 0.9 nM; Table 3) [33].

A number of spider toxins are promiscuous inhibitors of both K_v and Na_v channels, and therefore we also tested the effects of Aa1a and Ap1a on the human Na_v channels h Na_v 1.2, h Na_v 1.5 and h Na_v 1.7 which are found in the CNS, heart and peripheral sensory neurons, respectively (Fig. 5). Both peptides had about 11-fold selectivity for hEAG1 over Na_v 1.5, and moderate 2-fold selectivity over Na_v 1.2. However, while Aa1a has about 2-fold selectivity for hEAG1 over Na_v 1.7, Ap1a is approximately equipotent on these channels (Fig. 5C).

3.7 Ap1a and Aa1a are highly stable in serum and CSF

One of the major concerns for use of peptides *in vivo*, either as pharmacological tools or drugs, is their biological stability. However, the ICK fold typically provides venom peptides with high levels of chemical and thermal stability as well as resistance to proteases [26, 34]. Consistent with these observations, we found that Aa1a and Ap1a are highly stable in both human serum and CSF (Fig. 6). Ap1a remained intact after 3 days at 37°C in both fluids, and only 20% of Aa1a was degraded. Both peptides were not only much more stable than human atrial natriuretic peptide (hANP) but also significantly more resistant to degradation than the analgesic drug ziconotide (ω -conotoxin MVIIA), a venom-derived ICK peptide that is administered intrathecally [35] (Fig. 6).

4. Discussion

Despite abundant hEAG1 expression in the CNS, its neurophysiological role remains unclear [2]. hEAG1 has been shown to regulate neurotransmitter release and synaptic transmission [36] and gain-of-function mutations in hEAG1 lead to developmental abnormalities and infantile-onset epilepsy, such as in Temple-Baraitser and Zimmermann-Laband syndromes [5, 6, 37, 38]. Moreover, the *KCNH1* gene is predicted to be highly intolerant to functional genetic variation [39]. Thus, hEAG1 appears to be intimately involved in fundamental neuronal and developmental processes.

In this study we examined arachnid venoms as a potential source of hEAG1 inhibitors that could be used to probe the function of this enigmatic channel. We isolated two paralogous spider-

venom peptides, Aa1a and Ap1a, that inhibit hEAG1 with submicromolar potency. Both peptides are significantly more potent than the only two venom peptides previously reported to inhibit hEAG1, namely the sea anemone toxin APETx4 (IC_{50} 1,010 nM) and the scorpion toxin κ -hefutoxin (IC_{50} 26 μ M) (Table 3). Ap1a (IC_{50} 236 nM) is nearly as potent as astemizole, the most potent hEAG1 inhibitor described to date (IC_{50} 196 nM). Moreover, whereas Ap1a has advantageous 35-fold selectivity for hEAG1 over the cardiac channel hERG, astemizole is 200-fold *more potent* on hERG (IC_{50} 0.9 nM; Table 3). Astemizole was introduced in 1977 as a second-generation antihistamine but it was subsequently withdrawn from the market as the potent block of hERG by both astemizole and its major metabolite desmethyastemizole [33] can lead to potentially fatal QT prolongation [32]. Thus, non-selective small molecule hERG/hEAG1 blockers such as astemizole have limited therapeutic potential and they are not useful for studying hEAG1 function. While imipramine is commonly used to study hEAG1 *in vitro*, it is not a useful *in vivo* probe as it has only 2-fold selectivity over hERG (Table 3).

Ap1a has several other properties that recommend it as a useful tool for studying hEAG1 function *in vivo*. First, it has 11-fold selectivity over the cardiac $Na_v1.5$ channel in addition to its selectivity over hERG. Second, Ap1a and Aa1a are both highly stable in human serum and CSF, most likely due to their disulfide-rich ICK architecture. Thus, their *in vivo* half-life is likely to be primarily determined by the rate at which they are cleared by the liver and kidneys [40].

Ap1a does have some potentially undesirable pharmacological properties. It has only 2.2-fold selectivity for hEAG1 over $Na_v1.2$, which is primarily located in axon initial segments of excitatory neurons in the brain [41], and this might limit its utility for *in vivo* studies of the CNS population of hEAG1. Ap1a is also equipotent on $Na_v1.7$ and hEAG1. $Na_v1.7$ is an analgesic target that is largely confined to peripheral sensory neurons [42], and therefore block of this channel *in vivo* is unlikely to be problematic. However, given the promiscuous pharmacology of many venom peptides [43], future studies should examine the activity of Ap1a on a wider range of voltage- and ligand-gated ion channels.

Ap1a and Aa1a have complex effects on hEAG1 gating. Firstly, they shift the voltage dependence of channel activation in a depolarising direction (~ 20 mV at concentrations corresponding to their IC_{50}), which would cause channel open probability to drop by more than 50% during the repolarisation phase of the neuronal action potential (Fig. 4A). Although hEAG1 mutations that cause infantile-onset epilepsy are widely dispersed throughout the channel, they typically cause a hyperpolarising shift in the voltage-dependence of activation [5, 6]. Thus, gating modifiers such as

Aa1a and Ap1a that counteract the deleterious effect of these mutations by shifting the activation threshold to more depolarised potentials may prove to be better therapeutic candidates than pore blockers or gene-knockdown strategies for treating these epileptic disorders.

Secondly, Ap1a and Aa1a affect channel inactivation. Unlike hERG channels, which display an inward-rectifying I-V relationship due to fast and voltage-dependent C-type inactivation [44, 45], mammalian EAG channels are characterised by little or no voltage-dependent inactivation [3, 46]. Interestingly, we found that when the membrane was highly depolarised (i.e., potentials $> +80$ mV), a voltage- and time-dependent slow inactivation emerged during the activating step in a single-pulse protocol (Fig. 4A). This observation supports the hypothesis that hEAG1 channels have intrinsic slow inactivation [46], although mouse EAG1 channels do not exhibit this type of inactivation [47]. This time-dependent slow inactivation of hEAG1 was not evident at lower membrane potentials likely due to its very slow kinetics. Ap1a caused this slow open-state inactivation to occur at less depolarised membrane potentials ($> +50$ mV) and the effect became more significant as the membrane potential became more depolarised (Fig. 4A, right). This is reminiscent of the significant open-state inactivation of rEAG1 caused by partial [31] or complete [48] deletion of the EAG domain, or the inactivation of hEAG1 caused by the binding of 3-nitro-*N*-(4-phenoxyphenyl)-benzamide (ICA105574) or a Y464A mutation in the S6 helix [46]. For mechanistic reasons that remain to be determined, Aa1a did not affect open-state inactivation of hEAG1 in the same manner as Ap1a, which might partially account for its lower potency.

Steady-state inactivation of hEAG1, reported to be 5–10% when tested at +30 mV after a prepulse to +20 mV [46], was more significant with the double-pulse protocol used in this study (Fig. 4C,D, right). This inactivation was largely countered by Aa1a and Ap1a (Fig. 4D, right). This steady-state inactivation appears to be different to the slow open-state inactivation discussed above, as Ap1a attenuated the former but promoted the latter, while Aa1a had more significant effects on the former than Ap1a, but no effects on the latter (Fig. 4). In this regard, the peptides are different to ICA105574, which dramatically promotes both steady-state and open-state inactivation [46]. The underlying mechanisms remain to be elucidated, but since the 10- and 12-state Markov models built upon experimental data could not exclude the likelihood that hEAG1 can inactivate from any state [46], it is possible that the peptides work on different inactivation states that pair with different closed or open states.

The proposed binding site for ICA105574 on hEAG1 is located in the pore module between two

adjacent subunits of the homotetrameric channel [49]. However, the cryo-EM structure of rEAG1 [1] revealed two glycosylation sites in the turret area, suggesting that the attached sugar chains might surround the extracellular opening to the pore and prevent binding of large toxins. It is therefore not surprising that both Aa1a and Ap1a are gating modifiers that likely bind to extracellular regions of the voltage sensing domain to exert influence on the pore domain, as demonstrated for many other gating modifier toxins [50].

In summary, we isolated and characterised two spider-venom peptides (Aa1a and Ap1a) that potently inhibit hEAG1 and have good selectivity over the closely related hERG channel. These peptides possess an ICK fold and are highly stable in biological fluids. Along with astemizole, Ap1a is the most potent hEAG1 inhibitor reported to date, making it an excellent pharmacological tool for studies of this enigmatic channel. It will be interesting in future studies to examine the therapeutic potential of these peptides in tumours that ectopically overexpress hEAG1 and in epileptic disorders caused by gain-of-function mutations in the channel.

Acknowledgements

We thank Prof. Steve Petrou (Florey Institute for Neuroscience and Mental Health, Melbourne, Australia) for the hEAG1 cell line, Dr Cas Simons (The University of Queensland, Brisbane, Australia) for the hKCNH1-pcDNA3.1 plasmid, and Prof. Jamie Vandenberg (Victor Chang Cardiac Research Institute, Sydney, Australia) for the hERG clone. We thank members of the Deutsche Arachnologische Gesellschaft (DeArGe), particularly Hans-Werner Auer, Arnd Schlosser and Reti Erler, for providing arachnids for milking. This research was facilitated by access to the Australian Proteome Analysis Facility supported under the Australian Government's National Collaborative Research Infrastructure Strategy (NCRIS). We acknowledge financial support from the National Health & Medical Research Council (Principal Research Fellowship to G.F.K., CJ Martin Fellowship to L.M., and Career Development Fellowship to J.J.M.) and The University of Queensland (Early Career Researcher Grant to L.M.).

Conflicts of Interest

None.

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Figure legends

Figure 1: Identification of venom peptides that inhibit hEAG1. **(A)** Crude venoms (5 μ l) from the theraphosid spiders *Avicularia aurantiaca* and *A. purpurea* robustly inhibited currents from hEAG1 channels expressed in *Xenopus* oocytes (panel **B**). **(C)** Chromatograms resulting from RP-HPLC fractionation of crude *A. aurantiaca* and *A. purpurea* venom using an Aeris Widespore C18 column (Phenomenex) using a gradient of solvent B (dashed blue line) and a flow rate of 0.7 ml min⁻¹. Absorbance was monitored at 214 nm and 280 nm (black and red traces, respectively). Fractions were manually collected and screened for hEAG1 inhibition. Fractions found to inhibit hEAG1 are shaded gold. **(D)** Aa1a and Ap1a were purified to homogeneity using a second round of separation on either a Hypersil C18 column (Aa1a, left) or a Pursuit 3 Diphenyl C18 column (Ap1a, right). **(E)** MALDI-TOF MS analysis revealed that these venom peptides have monoisotopic masses of 4148.217 Da (Aa1a, left) and 4220.077 Da (Ap1a, right). **(F)** The isolated peptides recapitulated the hEAG1 inhibitory activity of the corresponding crude venoms.

Figure 2: Sequence and structure of hEAG1 inhibitors. **(A)** Sequence alignment of Aa1a and Ap1a, with identical residues shaded blue. Disulfide bonds are indicated by orange lines. The secondary structure of Aa1a, identified by NMR structural analysis, is shown above the sequences. **(B)** Comparison of RP-HPLC elution times of native and synthetic peptides. Left panel shows analytical RP-HPLC chromatograms for elution of synthetic Aa1a, native Aa1a, and a mixture of synthetic and native Aa1a. Right panel shows analytical RP-HPLC chromatograms for elution of synthetic Ap1a, native Ap1a, and a mixture of synthetic and native Ap1a. Peptides were eluted from a Hypersil GOLD C18 column heated to 40°C using a gradient of 10–50% solvent B over 30 min and a flow rate of 0.3 ml min⁻¹, with detection at 214 nm. **(C)** Solution structure of Aa1a. Overlay of the ensemble of 20 Aa1a structures. β -Strands are coloured cyan and disulfide bonds are shown as orange tubes. **(D)** Ensemble of Aa1a structures showing the ladder of hydrophobic residues (F6, L7, W9, W29, and W32) aligned along one face of the peptide.

Figure 3: Potency and reversibility of hEAG1 inhibition by Aa1a and Ap1a. Representative TEVC traces showing inhibition of hEAG1 currents by increasing concentrations of **(A)** Aa1a and **(B)** Ap1a. **(C)** Fitting of a Hill equation to concentration-response curves for inhibition of hEAG1 yielded IC₅₀ values of 637 $\pm$ 58.6 nM (Hill coefficient 1.0 $\pm$ 0.1, n = 5) for Aa1a and 235.8 $\pm$ 21 nM (Hill coefficient 1.3 $\pm$ 0.1, n = 7) for Ap1a. **(D,E)** Representative traces showing the current changes caused by increasing concentrations of peptide, followed by a wash step. The peptide concentrations, which are the same as shown in panels A and B, are indicated in units of nM on

the figure. The red curve in panel (D) is a single-exponential fit to the wash-out of Aa1a, which yielded an off-rate of 19.7 ± 4.2 s ($n = 4$). Wash out of Ap1a (panel E) was too slow to allow estimation of the dissociation rate.

Figure 4: Effect of Aa1a and Ap1a on hEAG1 gating. (A) Left: Representative whole-cell currents, obtained using the activation protocol shown in the inset, showing voltage-dependent activation of hEAG1 channels expressed in CHO-K1 cells. Right: Activation current-voltage (I-V) relationships were built from normalised plateaued currents $I/I_{\max}$ (where I is the current at each test voltage and $I_{\max}$ is the maximum current observed for that cell) and fitted with the Boltzmann equation. Aa1a (600 nM) and Ap1a (200 nM) shifted the half-activation voltage ($V_{1/2}$) from 20.8 ± 4.5 mV ($n = 14$) to 39.2 ± 4.7 mV ($n = 7$) and 37.7 ± 3.1 mV ($n = 9$), respectively. (B) Left: Deactivation of hEAG1 was analyzed using the tail current at -140 mV following an activating pre-pulse to $+80$ mV (left). Right: The time constant of deactivation (τ) was obtained by fitting the deactivation curve with a single-exponential function. The time constant for hEAG1 deactivation (2.4 ± 0.6 ms, $n = 6$) was not significantly altered in the presence of 600 nM Aa1a (3.8 ± 0.8 ms, $n = 6$) or 200 nM Ap1a (3.0 ± 0.7 ms, $n = 6$). (C) Steady-state inactivation of hEAG1 was examined at $+40$ mV after 10 s pre-conditioning steps from -130 mV to $+80$ mV in 15-mV increments. Representative whole-cell currents (left) show the dependence of steady-state inactivation on the pre-conditioning membrane potential. The expanded view at right highlights the markedly different activation kinetics at $+40$ mV obtained with different pre-conditioning membrane potentials (Cole-Moore shift). The bottom panels are representative whole-cell currents illustrating the effect of the peptides on hEAG1 inactivation. (D) The effects of Aa1a and Ap1a on the Cole-Moore shift and steady-state inactivation of hEAG1 were reflected by the activation time constants (τ) at $+40$ mV after a -120 mV pre-conditioning step (left) and the I-V relationships at the start (middle) and end (right) of the $+40$ mV activation test pulse.

Figure 5: Effect of Aa1a and Ap1a on hERG and Na_v channels. (A,B) Relative potency of Aa1a (panel A) and Ap1a (panel B) on hEAG1, hERG and selected Na_v channels ($\text{Na}_v1.2$, $\text{Na}_v1.5$ and $\text{Na}_v1.7$) measured using patch-clamp electrophysiology. (C) IC_{50} values obtained by fitting a Hill equation to the concentration-response curves shown in panels A and B ($n = 3-8$).

Figure 6: Stability of Aa1a (2 μM) and Ap1a (2 μM) in human CSF (left) and plasma (right) relative to human atrial natriuretic peptide (hANP, 1 μM) and the analgesic drug ω -conotoxin MVIIA (MVIIA, 1 μM).

Table 1: List of arachnid venoms screened against hEAG1.

SPIDERS (ORDER ARANEAE)						
No.	Family	Genus	Species	Sex ¹	Continent ²	hEAG1 activity ³
1	Araneidae	<i>Eriophora</i>	<i>transmarina</i>	F	AUS	NA
2	Araneidae	<i>Nephila</i>	<i>pilipes</i>	F	AS	NA
3	Atracidae	<i>Hadronyche</i>	<i>infensa</i>	F	AUS	Inhibitor
4	Austrochilidae	<i>Hickmania</i>	<i>troglodytes</i>	F	AUS	Inhibitor
5	Ctenidae	<i>Ancylometes</i>	<i>rufus</i>	F	AM	NA
6	Ctenidae	<i>Ancylometes</i>	<i>spec. (Guatemala)</i>	F	AM	Inhibitor
7	Ctenidae	<i>Cupiennius</i>	<i>salei</i>	F	AM	NA
8	Ctenidae	<i>Phoneutria</i>	<i>fera</i>	F	AM	Inhibitor
9	Ctenidae	<i>Phoneutria</i>	<i>nigriventer</i>	F	AM	NA
10	Ctenidae	<i>Phoneutria</i>	<i>reidy</i>	F	AM	Agonist
11	Ctenidae	<i>Viridasius</i>	<i>fasciatus</i>	F	AM	NA
12	Dipluridae	<i>Diplura</i>	<i>spec. (Peru)</i>	F	AM	NA
13	Dipluridae	<i>Linothele</i>	<i>fallax</i>	F	AM	Agonist
14	Dipluridae	<i>Linothele</i>	<i>megatheloides</i>	F	AM	Inhibitor
15	Macrothelidae	<i>Macrothele</i>	<i>gigas</i>	F	AS	NA
16	Nemesiidae	<i>Acanthogonatus</i>	<i>francki</i>	F	AM	Inhibitor
17	Nemesiidae	<i>Acanthogonatus</i>	<i>pissii</i>	F	AM	Inhibitor
18	Pisauridae	<i>Megadolomedes</i>	<i>australianus</i>	F	AUS	Inhibitor
19	Sparassidae	<i>Holconia</i>	<i>murrayensis</i>	F	AUS	Agonist
20	Sparassidae	<i>Isopeda</i>	<i>villosa</i>	F	AUS	Inhibitor
21	Sparassidae	<i>Neosparassus</i>	<i>diana</i>	F	AUS	NA
22	Sparassidae	<i>Polybetes</i>	<i>pythagoricus</i>	F	AM	NA
23	Sparassidae	<i>Sparassidae</i>	<i>spec. (Flores, Indonesia)</i>	F	AS	Agonist
24	Theraphosidae	<i>Acanthoscurria</i>	<i>geniculata</i>	F	AM	NA
25	Theraphosidae	<i>Augacephalus</i>	<i>ezendami</i>	F	AF	NA
26	Theraphosidae	<i>Avicularia</i>	<i>aurantiaca</i>	F	AM	Inhibitor*
27	Theraphosidae	<i>Avicularia</i>	<i>cf juruensis</i>	F	AM	Inhibitor*
28	Theraphosidae	<i>Avicularia</i>	<i>purpurea</i>	F	AM	Inhibitor*
29	Theraphosidae	<i>Avicularia</i>	<i>spec. (Puerto Maldonado, Peru)</i>	F	AM	Inhibitor*
30	Theraphosidae	<i>Avicularia</i>	<i>variegata</i>	F	AM	Inhibitor*
31	Theraphosidae	<i>Brachypelma</i>	<i>boehmei</i>	F	AM	NA
32	Theraphosidae	<i>Caribena</i>	<i>versicolor</i>	F	AM	Inhibitor*
33	Theraphosidae	<i>Ceratogyrus</i>	<i>marshalli</i>	F	AF	Agonist
34	Theraphosidae	<i>Chaetopelma</i>	<i>olivaceum</i>	F	AF	NA
35	Theraphosidae	<i>Chilobrachys</i>	<i>dyscolus</i>	F	AS	NA
36	Theraphosidae	<i>Chromatopelma</i>	<i>cyaneopubescens</i>	F	AF	NA
37	Theraphosidae	<i>Coremiocnemis</i>	<i>tropix</i>	F	AUS	NA
38	Theraphosidae	<i>Cyriopagopus</i>	<i>hainanus</i>	F	AS	NA
39	Theraphosidae	<i>Encyocratella</i>	<i>olivacea</i>	F	AF	Inhibitor
40	Theraphosidae	<i>Heteroscodra</i>	<i>maculata</i>	F	AF	NA
41	Theraphosidae	<i>Homoeomma</i>	<i>spec. ("blue")</i>	F	AM	NA
42	Theraphosidae	<i>Hysterochrates</i>	<i>ederi</i>	F	AF	Inhibitor*
43	Theraphosidae	<i>Hysterochrates</i>	<i>gigas</i>	F	AF	NA
44	Theraphosidae	<i>Hysterochrates</i>	<i>hercules</i>	F	AF	NA
45	Theraphosidae	<i>Hysterochrates</i>	<i>spec (West Cameroon)</i>	F	AF	Inhibitor*
46	Theraphosidae	<i>Lampropelma</i>	<i>nigerrimum</i>	F	AS	NA
47	Theraphosidae	<i>Lasiodora</i>	<i>klugi</i>	F	AM	NA
48	Theraphosidae	<i>Monocentropus</i>	<i>lambertoni</i>	F	AF	NA
49	Theraphosidae	<i>Ornithoctonus</i>	<i>aureotibialis</i>	F	AS	Inhibitor
50	Theraphosidae	<i>Orphnaecus</i>	<i>spec. ("treedweller")</i>	F	AS	Inhibitor
51	Theraphosidae	<i>Pamphobeteus</i>	<i>insignis</i>	F	AM	NA
52	Theraphosidae	<i>Phormingochilus</i>	<i>carpenteri</i>	F	AS	Agonist

53	Theraphosidae	<i>Poecilotheria</i>	<i>fasciata</i>	F	AS	NA
54	Theraphosidae	<i>Psalmopoeus</i>	<i>cambridgei</i>	F	AM	Agonist
55	Theraphosidae	<i>Pterinochilus</i>	<i>murinus</i>	F	AF	NA
56	Theraphosidae	<i>Selenocosmia</i>	<i>crassipes</i>	F	AUS	NA
57	Theraphosidae	<i>Selenotholus</i>	<i>cf foelschei</i>	F	AUS	NA
58	Theraphosidae	<i>Sericopelma</i>	<i>spec. (Panama)</i>	F	AM	NA
59	Theraphosidae	<i>Stromatopelma</i>	<i>calceatum</i>	F	AF	NA
60	Theraphosidae	<i>Tapinauchenius</i>	<i>elenae</i>	F	AM	NA
61	Theraphosidae	<i>Thrixopelma</i>	<i>ockerti</i>	F	AM	NA
62	Theraphosidae	<i>Xenesthis</i>	<i>spec. ("white", Colombia)</i>	F	AM	NA
63	Theraphosidae	<i>Ybyrapora</i>	<i>diversipes</i>	F	AM	Inhibitor*
SCORPIONS (ORDER SCORPIONES)						
No.	Family	Genus	Species	Sex ¹	Continent ²	hEAG1 activity ³
64	Bothriuridae	<i>Bothriurus</i>	<i>coriaceus</i>	U	AM	Inhibitor
65	Bothriuridae	<i>Bothriurus</i>	<i>dumayi</i>	U	AM	Agonist
66	Bothriuridae	<i>Bothriurus</i>	<i>keyserlingi</i>	U	AM	Inhibitor
67	Buthidae	<i>Androctonus</i>	<i>amoureuxi</i>	M	AF	Agonist
68	Buthidae	<i>Androctonus</i>	<i>australis</i>	U	AF	Agonist
69	Buthidae	<i>Androctonus</i>	<i>australis "Hector"</i>	M	AF	Inhibitor
70	Buthidae	<i>Androctonus</i>	<i>bicolor</i>	U	AF	NA
71	Buthidae	<i>Androctonus</i>	<i>crassicauda</i>	U	AF	Inhibitor
72	Buthidae	<i>Androctonus</i>	<i>crassicauda</i>	M	AF	Inhibitor
73	Buthidae	<i>Androctonus</i>	<i>mauritanicus</i>	U	AF	NA
74	Buthidae	<i>Apistobuthus</i>	<i>pterygocercus</i>	F	AF	NA
75	Buthidae	<i>Centruroides</i>	<i>bicolor</i>	U	AM	NA
76	Buthidae	<i>Hottentotta</i>	<i>franzwernerii</i>	M	AF	NA
77	Buthidae	<i>Hottentotta</i>	<i>gentili</i>	M	AS	NA
78	Buthidae	<i>Hottentotta</i>	<i>jayakari</i>	F	AF	Agonist
79	Buthidae	<i>Hottentotta</i>	<i>judaicus</i>	F	AF	NA
80	Buthidae	<i>Hottentotta</i>	<i>minusalta</i>	F	AS	NA
81	Buthidae	<i>Hottentotta</i>	<i>salei</i>	U	AF	Agonist
82	Buthidae	<i>Leiurus</i>	<i>abdullahbayrami</i>	M	AF	Inhibitor
83	Buthidae	<i>Leiurus</i>	<i>cf haenggii</i>	F	AF	NA
84	Buthidae	<i>Leiurus</i>	<i>hebraeus</i>	F	AF	NA
85	Buthidae	<i>Leiurus</i>	<i>quinquestriatus</i>	U	AF	Inhibitor
86	Buthidae	<i>Leiurus</i>	<i>spec. (Oman)</i>	U	AS	NA
87	Buthidae	<i>Leiurus</i>	<i>spec. ("ullrichi")</i>	F	AF	Agonist
88	Buthidae	<i>Mesobuthus</i>	<i>gibbosus</i>	U	EU	NA
89	Buthidae	<i>Parabuthus</i>	<i>capensis</i>	M	AF	Inhibitor
90	Buthidae	<i>Parabuthus</i>	<i>mossambicensis</i>	F	AF	Agonist
91	Buthidae	<i>Parabuthus</i>	<i>raudus</i>	U	AF	NA
92	Buthidae	<i>Parabuthus</i>	<i>schlechteri</i>	U	AF	NA
93	Buthidae	<i>Parabuthus</i>	<i>stridulus</i>	M	AF	NA
94	Buthidae	<i>Parabuthus</i>	<i>transvaalicus</i>	F	AF	NA
95	Buthidae	<i>Parabuthus</i>	<i>villosus</i>	U	AF	NA
96	Buthidae	<i>Parabuthus</i>	<i>villosus ("black")</i>	M	AF	NA
97	Caraboctonidae	<i>Hadrurus</i>	<i>arizonensis</i>	U	AM	Agonist
98	Scorpionidae	<i>Heterometrus</i>	<i>bengalensis</i>	U	AS	Agonist
99	Scorpionidae	<i>Heterometrus</i>	<i>cf cyaneus</i>	U	AS	Inhibitor
100	Scorpionidae	<i>Heterometrus</i>	<i>cyaneus</i>	U	AS	Agonist
101	Scorpionidae	<i>Heterometrus</i>	<i>petersii</i>	U	AS	Agonist
102	Scorpionidae	<i>Heterometrus</i>	<i>spinifer</i>	U	AS	NA
103	Scorpionidae	<i>Nebo</i>	<i>hierichonticus</i>	U	AF	Inhibitor
104	Scorpionidae	<i>Nebo</i>	<i>omanensis</i>	F	AF	Inhibitor
105	Scorpionidae	<i>Nebo</i>	<i>whitei</i>	F	AF	NA

106	Scorpionidae	<i>Opisthophthalmus</i>	<i>boehmi</i>	M	AF	NA
107	Scorpionidae	<i>Pandinoidea</i>	<i>cavimanus</i>	U	AF	Agonist
108	Vaejovidae	<i>Paravaejovis</i>	<i>hoffmanni</i>	U	AM	Inhibitor

Footnotes: ¹Sex: F, female; M, male; U, unknown; ²Continent: AM, America; AF, Africa; AS, Asia; AUS, Australia;

³Activity against hEAG1 in TEVC screen: venoms are defined as containing an inhibitor or agonist of hEAG1, or not active (NA) against this channel. The nine venoms that caused >50% of K_v10.1 are highlighted with an asterisk (*).

Table 2: Structural statistics for the ensemble of Aa1a structures (PDB ID 5WLX)¹

Experimental restraints	
<i>Inter-proton distance restraints</i>	
Total	523
Intra-residue ($i = j$)	76
Sequential ($ i - j = 1$)	144
Medium range ($1 < i - j < 5$)	120
Long range ($ i - j \geq 5$)	183
<i>Disulfide-bond restraints</i>	9
<i>Dihedral-angle restraints (ϕ, ψ)</i>	
Total	63
ϕ dihedral angle restraints	25
ψ dihedral angle restraints	24
χ^1 angle restraints	14
Total number of restraints per residue	16.5
Violations of experimental restraints	0
RMSD from mean coordinate structure (Å)	
All backbone atoms	0.74 ± 0.48
All heavy atoms	0.97 ± 0.42
Backbone atoms (Residues 3–34)	0.25 ± 0.08
Heavy atoms (Residues 3–34)	0.62 ± 0.09
Stereochemical quality²	
Residues in most favored Ramachandran region (%)	79.4 ± 3.3
Residues in disallowed Ramachandran regions (%)	0.0 ± 0.0
Favorable sidechain rotamers (%)	82.9 ± 5.2
Unfavorable sidechain rotamers (%)	4.8 ± 1.7
Clashscore, all atoms ³	0.0 ± 0.0
Overall MolProbity score	1.7 ± 0.1

¹ All statistics are given as mean $\pm$ S.D.

² Stereochemical quality according to MolProbity (<http://helix.research.duhs.duke.edu>).

³ Clashscore is defined the number of steric overlaps >0.4 Å per 1000 atoms.

Table 3: Pharmacological properties of hEAG1 inhibitors.

		IC ₅₀ (nM) or extent of channel inhibition (%)									Ref. ³
		hEAG1	hERG	K _V 1.3	K _V 1.4	K _V 2.1	Na _v 1.2	Na _v 1.4	Na _v 1.5	Na _v 1.7	
Small molecule	astemizole	196	0.9	ND ¹	ND	ND	ND	ND	ND	ND	1,2
	imipramine	1800	3400	ND	ND	ND	ND	ND	ND	ND	1
Venom peptide	Ap1a	236	8197	ND	ND	ND	519	ND	2565	222	3
	Aa1a	637	8511	ND	ND	ND	1449	ND	7316	1528	3
	APETx4	1010	20% at 10 μ M	27% at 1.6 μ M	66% at 1.6 μ M	39% at 1.6 μ M	ND	50% at 1.6 μ M	34% at 1.6 μ M	ND	4
	κ -hefutoxin	26000	NE ²	40000	NE	NE	ND	ND	ND	ND	5–7

¹ND = not determined; ²NE = no effect; ³References: 1. [12]; 2. [33]; 3. Current study; 4. [14]; 5. [51]. 6. [52]. 7. [53].