Abstract

The Kv1.3 voltage-gated potassium channel regulates membrane potential and calcium signaling in human effector memory T cells that are key mediators of autoimmune diseases such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis. Thus, subtype-specific Kv1.3 blockers have potential for treatment of autoimmune diseases. Several Kv1.3 channel blockers have been characterized from scorpion venom, all of which have an α/β scaffold stabilized by 3–4 intramolecular disulfide bridges. Chemical synthesis is commonly used for producing these disulfide-rich peptides but this approach is time consuming and not cost effective for production of mutants, fusion proteins, fluorescently tagged toxins, or isotopically labelled peptides for NMR studies. Recombinant production of Kv1.3 blockers in the cytoplasm of E. coli generally necessitates oxidative refolding of the peptides in order to form their native disulfide architecture. An alternative approach that avoids the need for refolding is expression of peptides in the periplasm of E. coli but this often produces low yields. Thus, we developed an efficient Pichia pastoris expression system for production of Kv1.3 blockers using margatoxin (MgTx) and agitoxin-2 (AgTx2) as prototypic examples. The Pichia system enabled these toxins to be obtained in high yield (12–18 mg/L). NMR experiments revealed that the recombinant toxins adopt their native fold without the need for refolding, and electrophysiological recordings demonstrated that they are almost equipotent with the native toxins in blocking Kv1.3 (IC50 values of 201±39 pM and 97±3 pM for recombinant AgTx2 and MgTx, respectively). Furthermore, both recombinant toxins inhibited T-lymphocyte proliferation. A MgTx mutant in which the key pharmacophore residue K28 was mutated to alanine was ineffective at blocking Kv1.3 and it failed to inhibit T-lymphocyte proliferation. Thus, the approach described here provides an efficient method of producing toxin mutants with a view to engineering Kv1.3 blockers with therapeutic potential.

Introduction

Voltage-gated potassium (Kv) channels are expressed in a wide range of cell types and tissues where they play key roles in physiological processes such as cell excitability, muscle contraction, and regulation of cardiac function [1]. Kv channels are composed of four α subunits that together form a functional channel [2]. There are nine subfamilies of Kv channels, with Kv1.3 being one of eight subtypes in the Kv1.x subfamily. Kv1.3 channels are strongly upregulated during the activation of human effector memory T (TEM) cells, which play a crucial role in the pathogenesis of autoimmune diseases such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis. The Kv1.3 channel has consequently become a target for drugs to treat autoimmune diseases [3–8]. ShK, a sea anemone peptide that potently and selectively blocks Kv1.3, was shown to be effective in six animal models of autoimmune disease; MS, TID, rheumatoid arthritis, allergic contact dermatitis, bone resorption and delayed type hypersensitivity [9]. ShK will soon enter Phase 1 clinical trials for treatment of autoimmune disease [10].

Peptides derived from animal venom are the largest source of ion channel blockers and they have proved to be a valuable resource for developing drugs to treat a variety of diseases [10,11]. Numerous peptidic Kv1.3 blockers have been isolated from scorpion venom [12,13], with members of the α-KTx subfamily of scorpion toxins displaying an extraordinary ability to distinguish between the large family of Kv channels and the maxi-K channel [14,15]. Peptides from the α-KTx subfamily contain 23–43 amino acid residues and they share a common structural motif comprising an α-helix and 2–3 antiparallel β-strands stabilized by 3–4 disulfide bridges.

Although α-KTx peptides inhibit Kv1.3 at sub-nanomolar concentrations, they often also inhibit other Kv1.x subtypes. Thus, in order for these toxins to have therapeutic application, their selectivity needs to be engineered to avoid the deleterious effects caused by off-target activity on other Kv subtypes. However,
progress in this area has been slow as isolation of toxins from crude venom yields minute amounts of material and chemical synthesis is time consuming and not cost-effective for production of mutant toxins for structure-activity relationship (SAR) studies. Recombinant production of Kv1.3 blockers in the cytoplasm of E. coli generally necessitates oxidative refolding of the peptides in order to form their native disulfide architecture [16]. An alternative approach that generally avoids the need for refolding is expression of peptides in the periplasm of E. coli [17–19] but this often results in low yields. Thus, it would be highly beneficial to develop an efficient and cost-effective method for production of these potent ion channel modulators.

We have developed an efficient Pichia pastoris expression system for production of peptide Kv1.3 channel blockers. We demonstrate the efficacy of this system via the production of recombinant agitoxin-2 (AgTx2) and margatoxin (rMgTx), Kv1.3 blockers isolated from the venom of the scorpions Centruroides margaritatus (Central American bark scorpion) and Leuotus quinquenestriatus helvus (Israeli yellow scorpion), respectively. These peptides are 46% identical at the amino acid level (Fig. 1). The 3D structures of AgTx2 (pdb: 1AGT) and MgTx (pdb: 1MTX) contain an α-helix anchored by three antiparallel β-strands stabilized by three disulﬁde bonds (Fig. 1) [20,21]. Both peptides were previously produced by overexpression in E. coli but, although the recombinant peptides were fully functional, the yields of recombinant MgTx and AgTx2 were less than 2 mg/L [20–22].

P. pastoris has proved to be an excellent host for heterologous expression of disulfide-rich peptides [23–25]. In the present study we show that both MgTx and AgTx2 can be produced in P. pastoris with yields 6–8-fold higher than previously reported for production in E. coli. The recombinant AgTx2 and MgTx produced in P. pastoris show high potency towards mouse Kv1.3 channels stably expressed in L929 mouse ﬁbroblast cells and they also inhibited proliferation of human T-lymphocytes.

Materials and Methods

Ethics statement

All experiments involving live animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Production of recombinant MgTx, AgTx2, and a K28A mutant of MgTx

Expression and puriﬁcation of MgTx, AgTx2, and a K28A mutant of MgTx

Expression and puriﬁcation of MgTx, AgTx2, and a K28A mutant of MgTx were performed using the protocols we described previously [25]. Synthetic genes encoding MgTx and AgTx2, with codons optimized for P. pastoris, were constructed by ligating four complementary and overlapping oligonucleotides coding for the protein sequences of MgTx (UniProt P40755) and AgTx2 (UniProt P46111). The MgTx gene was amplified using PCR with the sense primer:

5’-GAATTCATCATCATCATCATCATCATATTATTACGTTATXGTGTAC-3’ that has an EcoRI recognition site (underlined) and encodes six histidine residues (bold) to facilitate toxin puriﬁcation. The antisense primer was:

5’-CCGGGTATATGTTGGTAACACTTACACTTACCTGGTTACACTTGTT-3’ with a SacII recognition site (underlined) and a TTA stop codon (bold). The AgTx2 gene was ampliﬁed using PCR with the sense primer:

5’-GAATTCATCATCATCATCATCATCATGTTGTTCACATATAATGTTTCTGTG-3’ that has an EcoRI recognition site (underlined) and encodes six histidine residues (bold) to facilitate toxin puriﬁcation. The antisense primer was:

5’-CCGGGTATATGTTGGTAACACTTACACTTACCTGGTTACACTTGTT-3’ with the underlined nucleotides denote the site of mutation.

PCR products were puriﬁed and cloned into the EcoRI and SacII sites of the yeast transfer vector pPICZaA. The recombinant plasmid was transformed into E. coli DH5α cells and transformants were selected using an agar plate with low salt LB (1% tryptone +0.5% yeast extract +0.5% NaCl +1.5% agar; pH 7.0) and 25 μg/ml of the antibiotic phleomycin D1 (Zeocin; Invitrogen). After the clone was conﬁrmed by sequencing the insert, a total of 10 μg plasmid was linearized by digestion with SacI. The linearized construct was transformed into Pichia strain X33 using heat shock (Pichia EasyComp; Invitrogen). Transformants integrated at the 5′-AOX1 locus using single crossover, and colonies were selected using an agar plate with yeast peptone dextrose (YPD) (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and 100 μg/ml zeocin. For each construct, 6–10 clones were initially chosen, and those found to produce the highest levels of MgTx and AgTx2 based on SDS-PAGE gel analysis of small-scale test expressions were selected for large-scale toxigen expression.

We produced 15N-labelled MgTx and AgTx2 as follows: 100 μL of cell stock was grown at 30 °C in 200 ml of 15N minimal medium (0.34% yeast nitrogen base (YNB) without ammonium sulphate and amino acids, 2% dextrose, 0.02% biotin, and 0.05% 15NH4Cl) in 100 mM potassium phosphate buffer at pH 6.0 with 150 μg/ml zeocin for 40 h. The cells were then transferred into 800 ml of 15N minimal medium. After another 48 h, the OD600 was 25–30. The cells were then centrifuged, collected, and grown in 1 litre of 15N minimal methanol medium (0.34% YNB without ammonium sulphate and amino acids, 0.02% biotin, 1% methanol, and 0.05% 15NH4Cl). Once every 24 h, 1% methanol was added to induce protein expression for 2 days. The supernatant was collected by centrifugation, dialyzed twice against 5 litres of H2O.

Figure 1. Alignment of the primary structures of agitoxin-2 (AgTx2) and margatoxin (MgTx). Conserved residues are highlighted with a grey background and cysteine residues are shown in red. The lines above the sequences indicate disulfide bonds. The secondary structure of AgTx2 (based on PDB file 1AGT) is shown above the sequences.

doi:10.1371/journal.pone.0052965.g001
then loaded onto a nickel affinity column equilibrated with Equilibration Buffer (25 mM Tris, 150 mM NaCl, pH 8.0). After washing with Equilibration Buffer to remove nonspecific binders, the recombinant toxin was eluted with Elution Buffer (25 mM Tris, 150 mM NaCl, 200 mM imidazole, pH 8.0). Recombinant MgTx and AgTx2 were further purified by reverse-phase (RP) HPLC. RP-HPLC was performed on a Vydac C18 column (250 x 4.6 mm; particle size 5 μm) using a flow rate of 1 ml/min and a gradient of 20–30% solvent B (0.1% trifluoroacetic acid in 90% acetonitrile) in solvent A (0.1% trifluoroacetic acid in water) over 40 min. The purity of the recombinant toxins was judged using tricine SDS-PAGE [26].

**Mass spectrometry**

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry was performed using a Model 4700 Proteomics Bioanalyzer (Applied Biosystems, CA, USA). HPLC fractions were mixed (1:1, v:v) with α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50% H2O/50% acetonitrile containing 0.1% formic acid) and MALDI-TOF spectra were collected in positive reflector mode. All masses given are for the monoisotopic M+H+ ions.

**Western blots**

Proteins were separated under reducing conditions on a SDS-PAGE gel then transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare Australia, NSW, Australia) by electrolotting at 400 mA for 1 h. The membrane was then washed several times with PBS and blocked with Odyssey blocking buffer for 1 h. Then membrane was treated with anti-His antibody (His-probe Antibody H-15 rabbit polyclonal IgG, Santa Cruz Biotechnology, CA, USA) for 60 min at room temperature. After 4–5 washes, the membrane was treated with secondary antibody (IRDye® 800CW goat polyclonal anti-rabbit IgG, LI-COR Biosciences, NE, USA) for 60 min at room temperature. The membrane was then washed 4–5 times with PBS plus Tween 20 followed by a final wash with PBS to remove the detergent. The membrane was then scanned using an Odyssey infrared imaging system (LI-COR Biosciences).

**Cells and cell lines**

L929 mouse fibroblast cells stably expressing mouse Kv1.1 or mouse Kv1.3 channels [27] were kind gifts from Dr. George Chandy (University of California, Irvine). The cells were maintained in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 1% RPMI vitamins, 50 mM β-mercaptoethanol, and 1% heat-inactivated fetal bovine serum. T lymphocyte activation and proliferation was induced by the addition of 60 ng/ml anti-CD3 antibody (Clone OKT3, eBioscience, San Diego, CA, USA). Cells were cultured for 72 h at 37° C, 5% CO2, and [3H] thymidine was added during the last 16–18 h of culture. Cells were lysed by freezing at −20°C. After thawing, DNA was harvested onto fiberglass filters using a cell harvester (Inotech Biosystems International, Rockville, MD, USA). [3H] thymidine incorporation into the DNA of proliferating cells was measured using a β-scintillation counter (Beckman Coulter, Brea, CA, USA).

**NMR spectroscopy**

NMR spectra were acquired at 600 MHz using a Bruker Avance 600 NMR spectrometer at National Cheng Kung University. Samples were prepared in 10% D2O/90% H2O or 100% D2O, then the pH was adjusted with KOH to 4.6. Data were processed using XWINNMR and analyzed with Aurelia software. 2D 1H NMR spectra were recorded in the phase-sensitive absorption mode with quadrature detection in both F1 and F2 dimensions [28]. Samples of 2 mM 15N labelled MgTx and AgTx2 were used to acquire 2D 1H-15N HSQC spectra as well as 3D 15N edited TOCSY-HSQC and NOESY-HSQC spectra. Mixing times of 30–90 ms and 60–150 ms were used for TOCSY and NOESY experiments, respectively. The observed 1H chemical shifts were referenced with respect to the H2O or HOD signal, which was taken as 4.754 ppm downfield from external sodium 3-trimethylsilylpropionate-2,2,3,3-d4 (TSP) in D2O (0.0 ppm) at 300 K. The nitrogen chemical shift was referenced to external 15NH4Cl (3 mM in 1 M HCl) at 300 K, which is at 24.93 ppm downfield from liquid NH3. 1H-2H exchange rates for backbone amide protons were measured by recording DQF-COSY spectra 24 h after dissolving the protein in 100% D2O [29].

1H and 15N resonance assignments have been deposited in the Biological Magnetic Resonance Bank (www.bmrb.wisc.edu) under accession numbers BMRB-15420 (rMgTx) and BMRB-15421 (rAgTx2).

**Results**

**Expression, purification and characterization of MgTx and AgTx2**

AgTx2, MgTx, and MgTx-K28A were expressed using the pPICZαA vector in P. pastoris strain X-33. Each of the constructs encoded eight additional N-terminal amino acid residues (EFHHHHHHH). The EF residues are vestiges of the vector, while the His tag was used for affinity purification of the toxins. Western blots summarising the overexpression and purification of AgTx2 and MgTx are shown in Fig. 2. Analysis of the blots revealed that the culture supernatants contained predominantly the soluble recombinant peptides (Fig 2). The recombinant toxins were purified to homogeneity by nickel affinity chromatography...
followed by C18 RP-HPLC as shown in Fig. 3. The mass of both toxins estimated from the gel in Fig. 2 (∼7 kDa) was higher than expected, presumably because their high isoelectric point (pI ∼9.1 for both toxins) reduces their electrophoretic mobility. However, the masses of the purified recombinant proteins determined using mass spectrometry agreed well with the predicted masses for the fully oxidized peptides (MgTx: predicted = 5279.1 Da, experimental = 5277.6 Da; AgTx2 predicted = 5190.0 Da, experimental = 5190.0 Da; MgTx-K28A: predicted = 5218.36 Da, experimental = 5218.98). This indicated that, as expected, each of the recombinant toxins contains three disulfide bonds (Fig. 3). The yields of unlabeled rMgTx, rMgTx-K28A, and rAgTx2 produced in \textit{P. pastoris} were 12–15 mg/L, 8–10 mg/mL, and 14–18 mg/L, respectively. The yields of \textsuperscript{15}N-labeled MgTx and AgTx2 produced in high yield (8–10 mg/L). However, in contrast to rAgTx2 and rMgTx, rMgTx-K28A had no effect on \textit{m}KV1.1 currents at concentrations up to 100 nM (Fig. 4F). The mutant toxin blocked \textit{m}KV1.1 currents with an IC\textsubscript{50} of 216±24 nM, representing a more than 2,000-fold loss in activity on this channel relative to the native toxin (Fig. 4C, F). Consistent with this markedly reduced activity on \textit{m}KV1.1, the rMgTx-K28A mutant did not affect the proliferation of T lymphocytes (Fig. 4).

**Figure 3.** Chromatograms showing purification of (A) rAgTx2 and (B) rMgTx using HPLC. RP-HPLC was performed on a VyDAC C\textsubscript{18} column using a flow rate of 1 mL/min and a gradient of 20–30% acetonitrile over 40 min. The inset in each chromatogram is a MALDI-TOF spectrum showing the average mass of purified recombinant toxin. doi:10.1371/journal.pone.0052965.g003

**Figure 2.** Western blot using anti-His\textsubscript{6} primary antibody showing expression and purification of recombinant (A) AgTx2 and (B) MgTx produced in \textit{P. pastoris}. The mass (in kDa) of the prestained molecular weight markers (“Stds”) is indicated. Lane 1, culture supernatant after methanol induction of toxin expression; Lane 2, recombinant peptide after purification using nickel affinity chromatography; Lane 3 recombinant peptide after RP-HPLC purification. doi:10.1371/journal.pone.0052965.g002

**Figure 4.** Inhibition of mouse Kv1.3 and Kv1.1 by recombinant MgTx and AgTx2. AgTx2 and MgTx are reported to block K\textsubscript{v}1.3 and K\textsubscript{v}1.1 channels efficiently at micromolar concentrations [23,30,31]. We therefore measured the ability of rAgTx2 and rMgTx to block mouse K\textsubscript{v}1.3 (mK\textsubscript{v}1.3) and mouse K\textsubscript{v}1.1 (mK\textsubscript{v}1.1) channels stably expressed in L929 cells. As expected, rAgTx2 blocked both K\textsubscript{v}1.3 currents with equivalent potency (IC\textsubscript{50} = 201±59 pM on mK\textsubscript{v}1.3 and 144±30 pM on mK\textsubscript{v}1.1), with a Hill coefficient of 1 (Fig. 4A, D). rMgTx exhibited a higher affinity for mK\textsubscript{v}1.3 (IC\textsubscript{50} = 97±3 pM) than for mK\textsubscript{v}1.1 (IC\textsubscript{50} = 396±41 pM) (Fig. 4B, E). The recombinant rMgTx and rAgTx2 inhibited mK\textsubscript{v}1.3 with similar potency to the native toxins (200 pM and 30–100 pM for AgTx2 and MgTx, respectively; [31]).

We next tested the ability of each recombinant peptide to block the proliferation of freshly-isolated human T lymphocytes, which are sensitive to KV1.3 channel blockers [32]. Both rAgTx2 and MgTx inhibited the proliferation of T lymphocytes in a dose-dependent manner with an IC\textsubscript{50} of ∼40 nM for rAgTx2 (Fig. 4G) and ∼6 nM for rMgTx (Fig. 4H). These concentrations are within the range previously described for MgTx (∼5 nM) and AgTx2 (>5 nM) [33,34]. Thus the biological activities of rAgTx2 and rMgTx produced in \textit{P. pastoris} are similar to the native toxins.

**Production and characterization of rMgTx-K28A.** α-KTx scorpion toxins that block K\textsubscript{v} channels contain a conserved lysine residue that fits snugly into the pore of the channel and makes intimate contact with the selectivity filter [35]. Mutation of this residue (K27) in agitoxin causes a dramatic reduction in toxin binding and activity [36]. In order to test the utility of the \textit{P. pastoris} system for producing toxin mutants, we decided to examine the effect of mutating the corresponding lysine residue (K28) in MgTx. The mutant toxin rMgTx-K28A was produced in high yield (5–10 mg/L). However, in contrast to rAgTx2 and rMgTx, rMgTx-K28A had no effect on \textit{m}KV1.1 currents at concentrations up to 100 nM (Fig. 4F). The mutant toxin blocked mK\textsubscript{v}1.3 currents with an IC\textsubscript{50} of 216±24 nM, representing a more than 2,000-fold loss in activity on this channel relative to the native toxin (Fig. 4C, F). Consistent with this markedly reduced activity on \textit{m}KV1.1, the rMgTx-K28A mutant did not affect the proliferation of T lymphocytes (Fig. 4).

**Structural analysis of MgTx and AgTx2.** We acquired 2D and 3D NMR spectra of \textsuperscript{15}N-labeled rMgTx and rAgTx2 at the same pH as in previous studies [20,21]. The \textsuperscript{1}H and \textsuperscript{15}N chemical shifts observed in the \textit{H}-\textit{H} HSQC spectra of rMgTx and rAgTx2 are consistent with previously reported values (Fig. 5A, B). The \textsuperscript{1}H chemical shifts of the N-terminal Thr1 (\Delta\delta = −1.0 ppm) and Ile2 (\Delta\delta = −0.56 ppm) residues in MgTx, as well as Val2 (\Delta\delta = −0.15 ppm) in AgTx2, were shifted slightly upfield presumably due to the six extra N-terminal histidine residues. The similarity between the \textsuperscript{1}H and \textsuperscript{15}N chemical shifts we observed for recombinant MgTx and AgTx2 and those reported previously for native MgTx and AgTx2 are suggestive that the recombinant peptides have adopted the native fold. However, in order to confirm this, we performed additional experiments to determine the disulfide pairings and secondary structure of the recombinant toxins. We identified disulfide connectivities by searching for sets of slowly exchanging amide protons. Strip plots from the \textsuperscript{15}N-edited NOE-HSQC spectrum of MgTx (pH 4.6) clearly showed NOEs between H\textsubscript{\alpha} of Cys7 and H\textsubscript{\textit{b}} of Cys33 and H\textsubscript{\textit{a}} of Cys34, and H\textsubscript{\textit{a}} of Cys34 and H\textsubscript{\textit{b}} of Cys36 disulfide bonds in rMgTx and rAgTx2. Thus, the disulfide-bond connectivities in the recombinant peptides produced in \textit{P. pastoris} are identical to those in the native peptides [20,21].

The secondary structure of rMgTx and rAgTx2 was determined by identification of NOE patterns characteristic of β-strands and α-helices in 2D and 3D NOE spectra. The formation of antiparallel β-sheets was characterized by identification of H\textsubscript{\alpha} of H\textsubscript{\alpha}, H\textsubscript{\alpha} of H\textsubscript{\alpha}, and H\textsubscript{\alpha} of H\textsubscript{\alpha} NOEs connecting strands (Fig. 6) and the presence of slowly exchanging amide protons. Strip plots from the \textsuperscript{15}N-edited NOE-HSQC spectrum of MgTx (pH 4.6) clearly showed NOEs between H\textsubscript{\alpha} of Cys7 and H\textsubscript{\alpha} of Cys33; H\textsubscript{\alpha} of Lys28
and HN of Tyr37; Hα of Tyr37 and HN of Lys28; and between HN of Met30 and HN of Lys33 (Fig. 6A). Similarly, strip plots from the 15N-edited NOESY-HSQC spectrum of AgTx2 (pH 4.6) revealed NOEs between Hα of Cys8 and HN of Cys33; Hα of Lys27 and HN of Thr36; Hα of Thr36 and HN of Lys27; HN of Val2 and HN of Cys35; HN of Val6 and HN of Cys33; HN of Arg24 and HN of Thr36; HN of Met29 and HN of Lys32; and between HN of Lys27 and HN of His34 (Fig. 6B). Furthermore, non-labile backbone amide protons were identified in both toxins by their slow exchange rate in 1H/2H exchange experiments. Based on the identification of slowly exchanging backbone amide protons and the dipolar connectivities shown in Fig. 6, we were able to confirm the formation of triple stranded antiparallel β-sheets in each toxin (Fig. 7A, B). Thus, the combined NMR data reveals that the recombinant AgTx2 and MgTx produced in P. pastoris adopt the same 3D fold as the native toxins.

**Discussion**

### Kv1.3 as a therapeutic target

Kv channels have diverse physiological functions; they play important roles in cell excitability, muscle contraction, regulation of neuronal and cardiac electrical functions, and propagation of action potentials [1]. The Kv1.3 subtype is upregulated during activation of terminally differentiated human effector memory T cells, which are important mediators of autoimmune diseases such as MS, T1D, and rheumatoid arthritis [3–6]. This has led to the hypothesis that compounds that selectively target Kv1.3 channels could be used to treat autoimmune diseases [38]. Consistent with this idea, ShK, a sea anemone toxin that potently blocks Kv1.3, reverses symptoms of experimental autoimmune encephalomyelitis and suppresses delayed type hypersensitivity in rats [3]. A stabilized version of ShK is about to enter Phase I clinical trials for...
the treatment of multiple sclerosis [39]. Thus, there is considerable interest in characterizing other blockers of Kv1.3 and determining their molecular mechanism of action in order to facilitate the rational design of small-molecule blockers of this channel.

Many animal toxins have extremely high specificity and potency for their molecular target due to millions of years of evolutionary fine-tuning. These features have made animal toxins extremely valuable as pharmaceutical tools and as leads for the development of novel therapeutics [10,11,40]. Scorpions are one of the best studied venomous animals. Along with centipedes, they are the oldest venomous animals, with the earliest scorpion fossils dating back to the Silurian period ~430 Mya [41]. The predominant components of scorpion venoms are disulfide-rich peptides, most of which modulate the activity of a wide variety of ion channels, including Kv channels. 18 families of peptidic Kv blockers, comprising 75 different peptides, have been described from scorpion venoms [42]. These peptides, known as α-KTx toxins, are comprised of 20–40 amino acid residues and 3-4 disulfide bonds.

Some α-KTx toxins are of significant interest because they potently block the human Kv1.3 channel. For example, both MgTx (α-KTx 2:2) and AgTx2 (α-KTx 3.2) potently inhibit Kv1.3 by blocking the central ion-conducting pore [21,22,43]. However, despite their similar 3D fold and high sequence homology, the affinity of α-KTx toxins for Kv1.3 varies over six orders of magnitude from picomolar to micromolar. Moreover, many of these toxins inhibit a number of different subtypes Kv channels with high affinity, an undesirable attribute for peptides being considered for therapeutic use due to the potential for deleterious side-effects. Selectivity can be improved by engineering the peptides through truncation of specified regions or by mutating particular amino acid residues. For example, ADWX-1, a mutant of BmKTX (α-KTx 3.6) with three residue changes, exhibits 80-fold higher affinity towards Kv1.3 (IC50 1.89 pM) than the native peptide (IC50 of 150 pM) [44]. Clearly, a system that allowed efficient production of toxin analogs in order to develop structure-activity relationships (SARs) would facilitate future engineering efforts aimed at improving potency and selectivity against Kv1.3.

Development of a Pichia expression system for production of Kv1.3 blockers

There are three major strategies for obtaining venom peptides: classical biochemical isolation from venom, chemical synthesis, and recombinant production in a heterologous system [19]. Since most venomous animals are very small, bioassay-guided isolation from venom usually produces only a limited amount of native peptide, thus precluding detailed SAR studies. Since most of these peptides contain 35–70 amino acid residues that are connected by 3–4 disulfide bridges, chemical synthesis of these peptides can be challenging both in terms of synthetic efficiency and the need to oxidatively refold the synthetic material to produce the correct disulfide-bond isomer. In some cases, the synthetic efficiency can be improved by splitting the synthesis into smaller fragments that are subsequently joined by native chemical ligation [45]. However, chemical synthesis is expensive, especially if one wishes to incorporate stable isotopes for NMR studies.

Recombinant production is the most cost-effective method for producing disulfide-rich peptides. The most common host for heterologous expression of venom peptides is E. coli [46,47] but this often necessitates oxidative refolding of the peptides [48] as the redox environment of the E. coli cytoplasm is not favorable for disulfide-bond formation [16]. Higher yields of correctly folded toxin can often be obtained by exporting the peptides into periplasm of E. coli, where the cellular machinery for disulfide-bond formation is located [17–19]; however, the overall yield of recombinant peptide is typically less than 5 mg/liter. Recombinant expression of disulfide-rich peptides in eukaryotic expression systems has also been reported. For example, the spider-venom peptide PcTx1, a potent blocker of acid sensing ion channel 1a, was successfully produced in insect cell lines, albeit at a very low yield of 0.5 mg/liter [49].

An alternative host for heterologous expression of disulfide-rich venom peptides is P. pastoris, which offers several advantages compared to other expression systems: (i) P. pastoris grows faster to reach higher cell densities in simpler media; (ii) Pichia grows well in minimal medium, making it suitable for production of isotopically labelled peptides; (iii) expressed peptides can be secreted into the culture medium, which simplifies purification as Pichia secretes low
levels of endogenous proteins [23–25]. Pichia has proved to be an excellent system for production of disulfide-rich snake-venom peptides, achieving yields as high as 150 mg/liter [24]. Thus, we attempted to develop a Pichia expression system for efficient production of disulfide-rich Kv1.3 blockers, using AgTx2 and MgTx as prototypic examples.

Previously, both MgTx and AgTx2 were expressed in E. coli at final yields of 2–3 mg/L [21,22]. In the present study, using the Pichia expression system, we were able to produce these toxins with...
Figure 7. Secondary structure of recombinant (A) AgTx2 and (B) MgTx determined from NMR data. Dipolar connectivities observed in 2D NOESY spectra are shown as double-sided red arrows. Cross-strand hydrogen bonds inferred from 1H-2H exchange rates are depicted as dashed orange lines. Green lines indicate disulfide-bonds inferred from NOE connectivities. The shaded arrows indicate the direction (N→C) of the three β strands.
doi:10.1371/journal.pone.0052965.g007

References

1. Giangiacomo KM, Geralde Y, Mullmann TJ (2004) Molecular basis of α-KTX specificity. Toxicon 43: 877–886.
2. Coetzee WA, Amarillo Y, Chiu J, Chow A, Lau D, et al. (1999) Molecular diversity of K+ channels. Ann NY Acad Sci 860: 233–285.
3. Beeton C, Wulff H, Barbara J, Clot-Faybesse O, Pennington M, et al. (2001) Selective blockade of T lymphocyte K+ channels ameliorates experimental autoimmune encephalomyelitis, a model for multiple sclerosis. Proc Natl Acad Sci USA 98: 13942–13947.
4. Wulff H, Beeton C, Chandy KG (2003) Potassium channels as therapeutic targets for autoimmune diseases. Curr Opin Drug Discov Dev 6: 640–647.
5. Beeton C, Chandy KG (2005) Potassium channels, memory T cells, and multiple sclerosis. Neuroscientist 11: 550–562.
6. Azam P, Sankaranarayanan A, Homierick D, Griffey S, Wulff H (2007) Targeting effector memory T cells with the small molecule Kv1.3 blocker PAP-1 suppresses allergic contact dermatitis. J Invest Dermatol 127: 1419–1429.
7. Pereira LE, Villinger F, Wulff H, Sankaranarayanan A, Raman G, et al. (2007) Pharmacokinetics, toxicity, and functional studies of the selective Kv1.3 channel blocker 5-(4-phenoxypytox)psoralen in rhesus macaques. Exp Biol Med 232: 1330–1334.
8. Mathew MP, Beeton C, Garcia A, Chi V, Rangaraju S, et al. (2008) Imaging of effector memory T cells during a delayed-type hypersensitivity reaction and suppression by Kv1.3 channel blocker Immunity 29: 602–614.
9. Rangaraju S, Chi V, Pennington MW, Chandy KG (2009) Kv1.3 potassium channels as a therapeutic target in multiple sclerosis. Expert Opin Ther Targets 13: 909–924.
10. King GF (2011) Venoms as a platform for human drugs: translating toxins into therapeutics. Expert Opin Biol Ther 11: 1469–1484.
11. Lewis RJ, Garcia ML (2003) Therapeutic potential of venom peptides. Nat Rev Drug Discov 2: 790–802.
12. Leonard RJ, Garcia ML, Slaughter RS, Reuben JP (1992) Selective blockers of voltage-gated K+ channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. Proc Natl Acad Sci USA 89: 10094–10098.
13. Mouhaidi S, Visan V, Ananthakrishnan S, Wulff H, Andreotti N, et al. (2005) Kv1.3 channel types targeted by synthetic OSK1, a toxin from Orthocanus scrobiculosus scorpion venom. Biochem J 385: 95–104.
14. Poppini L, Selsko B, Giurda G (1999) Structure and functions of scorpion toxins affecting K+ channels. Perspect Drug Discov Design 15/16: 15–40.
15. Tytgat J, Chandy KG, Garcia ML, Gutman GA, Martin-Eauclaire MF, et al. (1999) A unified nomenclature for short-chain peptides isolated from scorpion venoms. m-KTx molecular subfamilies. Trends Pharmacol Sci 20: 444–447.

16. Tedford HW, Fletcher JJ, King GF (2001) Functional significance of the β hairpin in the invertebrate neurotoxin ω-atracotoxin-Hv1a. J Biol Chem 276: 26568–26576.

17. Saez NJ, Mobi M, Bieri M, Chassagnon IR, Malde AK, et al. (2011) A dynamic pharmacophore drives the interaction between psalmotoxin-1 and the putative drug target acid-sensing ion channel 1a. Mol Pharmacol 80: 796–808.

18. Vetter I, Davis JL, Rash LD, Anangi R, Mobi M, et al. (2011) Venomics: a new paradigm for natural products-based drug discovery. Amino Acids 40: 15–28.

19. Meng F, Cai TF, Li WY, Zhang H, Liu YB, et al. (2011) Expression of spider neurotoxic peptide huwentoxin-I in E. coli. PLoS ONE 6: e21608.

20. Johnson BA, Stevens SP, Williamson JM (1994) Determination of the three-dimensional structure of margatoxin by 1H, 13C, 15N triple-resonance nuclear magnetic resonance spectroscopy. Biochemistry 33: 15061–15070.

21. Anang R, Chiu-Yueh C, Chun-Ho C, Yi-Chun C, Chun-Chun C, et al. (2007) Expression of snake venom toxins in Pichia pastoris and characterization of APETx2, a specific inhibitor of acid sensing ion channel 3. Toxicon 56: 1388.

22. Garcia-Calvo M, Leonard RJ, Novick J, Stevens SP, Schmalhofer W, et al. (1995) Purification, characterization, and biosynthesis of margatoxin, a component of Centruroides margaritatus venom that selectively inhibits voltage-dependent potassium channels. J Biol Chem 269: 18686–18674.

23. Chen CY, Cheng CH, Chen YC, Lee JC, Chou SH, et al. (2006) Preparation of amino-acid-type selective isotope labeling of protein expressed in Pichia pastoris. Proteins 62: 279–287.

24. Anangi R, Chi-Yueh C, Chun-Ho C, Yi-Chun C, Chun-Chun C, et al. (2007) Expression of snake venom toxins in Pichia pastoris. Toxicon 49: 169–187.

25. Anangi R, Chen CC, Lin YW, Cheng YR, Cheng CH, et al. (2010) Expression in Pichia pastoris and characterization of APETx2, a specific inhibitor of acid sensing ion channel 3. Toxicon 56: 1308–1307.

26. Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368–379.

27. Grissmer S, Nguyen AN, Ayan J, Hanson DC, Mather RJ, et al. (1994) Pharmacological characterization of five closed voltage-gated K+ channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. Mol Pharmacol 45: 1227–1234.

28. Marion D, Wuthrich K (1983) Application of phase-sensitive two-dimensional correlated spectroscopy (COSY) for measurements of 2H–1H spin–spin coupling constants in proteins. Biochem Biophys Res Commun 113: 967–974.

29. Wuthrich K (1980) NMR of proteins and nucleic acids. New York: John Wiley & Sons.

30. Garcia ML, Garcia-Calvo M, Hidalgo P, MacKinnon R (1994) Purification and characterization of three inhibitors of voltage-dependent K+ channels as targets for specific immunomodulation. Trends Pharmacol Sci 25: 280–289.

31. Cahalan MD, Chandy KG (2009) The functional network of ion channels in T lymphocytes. Immunol Rev 231: 58–87.