ShK toxin: history, structure and therapeutic applications for autoimmune diseases

Shih Chieh Chang, Saumya Bajaj, K. George Chandy

Laboratory of Molecular Physiology, Infection Immunity Theme, Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

Stichodactyla toxin (ShK) is a 35-residue basic peptide from the sea anemone Stichodactyla helianthus that blocks a number of potassium channels. An analogue of ShK called ShK-186 or Dalazatide is in human trials as a therapeutic for autoimmune diseases.

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History

Stichodactyla helianthus is a species of sea anemone (Phylum:Cnidaria) belonging to the family Stichodactylidae. Helianthus comes from the Greek words Helios meaning sun, and anthos meaning flower, which corresponds to S. helianthus common name “sun anemone”. It is sessile and uses potent neurotoxins for defense against its primary predator, the spiny lobster.\(^{(1)}\) The venom contains, among other components, numerous ion channel-blocking peptides. Figure 1 shows key milestones in the discovery of ShK and its development into a therapeutic for autoimmune diseases.\(^{\,(2-46)}\) In 1995, a group led by Olga Castaneda and Evert Karlsson, isolated ShK, a potassium channel-blocking 35-residue peptide from S. helianthus.\(^{(4)}\) The same year, William Kem and his collaborator Michael Pennington, synthesized and folded ShK, and showed it blocked neuronal and lymphocyte voltage-dependent potassium channels.\(^{(5)}\) In 1996, Ray
Norton, determined the three-dimensional structure of ShK.\(^6\) In 2005-2006, George Chandy, Christine Beeton and Michael Pennington developed ShK-170 and ShK-186, selective blockers of \(K_v1.3\).\(^{20,21}\) ShK-186, now called Dalazatide, was advanced to human trials in 2015-2017 by Shawn Iadonato and Eric Tarcha, as the first-in-man \(K_v1.3\) blocker for autoimmune disease.\(^{46}\)

**Figure 1 here**

**Structure**

ShK is cross-linked by three disulfide bridges: Cys3-Cys35, Cys12-Cys28, and Cys17-Cys32 (Figure 2). The solution structure of ShK reveals two short \(\alpha\)-helices comprising residues 14-19 and 21-24; the N-terminal eight residues adopt an extended conformation, followed by a pair of interlocking turns that resemble a 3\(_{10}\) helix; the C-terminal Cys35 residue forms a nearly head-to-tail cyclic structure through a disulfide bond with Cys3.\(^{6,10,24,35,41-42}\) Figure 3 shows the three-dimensional structure of ShK with key residues, in addition to the structures of related peptides: MMP-23’s ShK domain, BmK1 (from the filarial worm), and ShK-192 (an analogue of ShK-186), and the homology model of ShK-EWSS.

**Figure 2 here**

**Figure 3 here**

**Phylogenetic relationships of ShK and ShK domains**

The SMART database at the EMBL (http://smart.embl-heidelberg.de/smart/do_annotation.pl?ACC=SM00254), as of May 2018, lists 3345 protein domains with structural resemblance to ShK in 1797 proteins (1 to 8 domains/protein), many in the worm *Caenorhabditis elegans* and venomous snakes.\(^{25-29}\) The majority of these domains are in metallopeptidases, whereas others are in prolyl 4-hydroxylases, tyrosinases, peroxidases, oxidoreductases, or proteins containing epidermal growth factor-like domains, thrombospondin-type repeats, or trypsin-like serine protease domains.\(^{25-29}\) The only human proteins containing ShK-like domains are MMP-23 (matrix metalloprotease 23) (Figure 3) and MFAP-2 (microfibril-associated glycoprotein 2).\(^{25-29}\) Table 1 compares the sequence of ShK with related peptides from other sea anemones, parasitic worms and human MMP-23’s ShK domain; conserved residues are highlighted. Figure 4 shows the sequence similarity tree of these peptides; the tree was generated by NCBI’s Constraint-based Multiple Alignment Tool (COBALT).

**Table 1 here**
Channel targets

The ShK peptide blocks potassium (K⁺) ion channels Kv1.1, Kv1.3, Kv1.6, Kv3.2 and Ca3.1 with nanomolar to picomolar potency, and has no effect on the HERG (Kv11.1) cardiac potassium channel (Table 2). The neuronal Kv1.1 channel and the T lymphocyte Kv1.3 channel are most potently inhibited by ShK.

ShK binding configuration in K⁺ channels

ShK and its analogues are blockers of the channel pore. They bind to all four subunits in the K⁺ channel tetramer by interacting with the shallow vestibule at the outer entrance to the channel pore (Figure 5). These peptides are anchored in the external vestibule by two key interactions. The first is lysine²², which protrudes into and occludes the channel's pore like a "cork in a bottle" and blocks the passage of potassium ions through the channel pore (Figures 3 and 5). The second is the neighboring tyrosine²³, which together with lysine²² forms a "functional dyad" required for channel block. Many K⁺ channel-blocking peptides contain such a dyad of a lysine and a neighboring aromatic or aliphatic residue. Some K⁺ channel-blocking peptides lack the functional dyad, but even in these peptides a lysine physically blocks the channel, regardless of the position of the lysine in the peptide sequence.

Additional interactions anchor ShK and its analogues in the external vestibule and contribute to potency and selectivity. For example, arginine¹¹ and arginine²⁹ in ShK interact with two aspartate³⁸⁶ residues in adjacent subunits in the mouse Kv1.3 external vestibule (corresponds to aspartate⁴³³ in human Kv1.3).

Table 2

ShK analogues that block the Kv1.3 channel

Several ShK analogues have been generated to enhance specificity for the Kv1.3 channel over the neuronal Kv1.1 channel and other closely related channels (Table 2).

- ShK-Dap²²: This was the first analogue that showed some degree of specificity for Kv1.3. The pore-occluding lysine²² of ShK is replaced by diaminopropionic acid (Dap) in ShK-Dap²². Dap is a non-natural lysine analogue with a shorter side chain length (2.5 Å from Cα) than lysine (6.3 Å). Dap²² interacts with residues further out in the external
vestibule in contrast to lysine\textsuperscript{22}, which interacts with the channel’s selectivity filter.\textsuperscript{[16]} As a consequence, the orientations of ShK and ShK-Dap\textsuperscript{22} in the external vestibule are significantly different.\textsuperscript{[16]} ShK-Dap\textsuperscript{22} exhibits >20-fold selectivity for K\textsubscript{v}1.3 over closely related channels in whole-cell patch clamp experiments,\textsuperscript{[10]} but in equilibrium binding assays it binds K\textsubscript{v}1.1-K\textsubscript{v}1.2 heterotetramers with almost the same potency as ShK, which is not predicted from the study of homotetrameric K\textsubscript{v}1.1 or K\textsubscript{v}1.2 channels.\textsuperscript{[19]}

- **ShK-F6CA**: Attaching a fluorescein to the N-terminus of the peptide via a hydrophilic AEEA linker (2-aminoethoxy-2-ethoxy acetic acid; mini-PEG) resulted in a peptide, ShK-F6CA (fluorescein-6-carboxyl), with 100-fold specificity for K\textsubscript{v}1.3 over K\textsubscript{v}1.1 and related channels.\textsuperscript{[18]} Attachment of a tetramethyl-rhodamine or a biotin via the AEEA linker to ShK’s N-terminus did not increase specificity for K\textsubscript{v}1.3 over K\textsubscript{v}1.1.\textsuperscript{[18]} The enhanced specificity of ShK-F6CA might be explained by differences in charge: F6CA is negatively charged; tetramethylrhodamine is positively charged; and biotin is neutral.\textsuperscript{[18]} Subsequent studies with other analogues suggest that the negatively charged F6CA likely interacts with residues on the turret of the K\textsubscript{v}1.3 channel as shown for ShK-192 and ShK-EWSS (Figure 5).\textsuperscript{[24,41]}

- **ShK-170, ShK-186, ShK-192 and ShK-EWSS**: Based on ShK-F6CA additional analogues were made. Attaching a L-phosphotyrosine to the N-terminus of ShK via an AEEA linker resulted in a peptide, ShK-170, with 100-1000-fold specificity for K\textsubscript{v}1.3 over related channels. ShK-186 [a.k.a. SL5; a.k.a. Dalazatide] is identical to ShK-170 except the C-terminal carboxyl is replaced by an amide. ShK-186 blocks K\textsubscript{v}1.3 with an IC\textsubscript{50} of 69 pM and exhibits the same specificity for K\textsubscript{v}1.3 over closely related channels as ShK-170 (Table 2).\textsuperscript{[46]} The L-phosphotyrosine of ShK-170 and ShK-186 rapidly gets dephosphorylated in vivo generating an analogue, ShK-198, with reduced specificity for K\textsubscript{v}1.3 (Table 2).\textsuperscript{[20,32,33]} To overcome this problem, ShK-192 and ShK-EWSS were developed. In ShK-192, the N-terminal L-phosphotyrosine is replaced by a non-hydrolyzable para-phosphonophenylalanine (Ppa), and methionine\textsuperscript{21} is replaced by the non-natural amino acid norleucine to avoid methionine oxidation (Figure 3, Table 2).\textsuperscript{[24,32]} In ShK-EWSS, the AEEA linker and L-phosphotyrosine are replaced by the residues glutamic acid (E), tryptophan (W) and two serines (S) (Figures 3 and 5, Table 2).\textsuperscript{[41]} Both ShK-192 and ShK-EWSS are highly specific for K\textsubscript{v}1.3 over related channels.

- **ShK-K18A**: Docking and molecular dynamics simulations on K\textsubscript{v}1.3 and K\textsubscript{v}1.1 followed by umbrella sampling simulations, paved the way to the selective Kv1.3 inhibitor ShK-K18A.\textsuperscript{[50]}
● **ShK-related peptides in parasitic worms:** AcK1, a 51-residue peptide from hookworms *Ancylostoma caninum* and *Ancylostoma ceylanicum*, and BmK1, the C-terminal domain of a metalloprotease from filarial worm *Brugia malayi*, adopt helical structures closely resembling ShK. AcK1 and BmK1 block Kv1.3 channels at nanomolar-micromolar concentrations, and they suppress rat effector memory T cells without affecting naive and central memory T cell subsets. Further, they suppress IFN-γ production by human T cells and they inhibit the delayed type hypersensitivity response caused by skin-homing effector memory T cells. *Teladorsagia circumcincta* is an economically-important parasite that infects sheep and goats. TcK6, a 90-residue protein with a C-terminal ShK-related domain, is upregulated during the mucosal dwelling larval stage of this parasite. TcK6 causes modest suppression of thapsigargin-triggered IFN-γ production by sheep T cells, suggesting that the parasite use this protein for immune evasion by modulating mucosal T cells.

**Extending circulating half-life of ShK peptides**

Due to their low molecular mass, ShK and its analogues are prone to rapid renal elimination. In rats, the half-life is ~6 min for ShK-186 and ~11 min for ShK-198, with a clearance rate of ~950 ml/kg-min. In monkeys, the half-life is ~12 min for ShK-186 and ~46 min for ShK-198, with a clearance rate of ~80 ml/kg-min.

● **PEGylation of ShK:** Conjugation of polyethylene glycol (PEG) to ShK[Q16K], an ShK analogue, increased its molecular mass and thereby reduced renal clearance and extended plasma half-life to 15 h in mice and 64 h in cynomolgus monkeys. PEGylation can also decrease immunogenicity and protect a peptide from proteolysis and non-specific adsorption to inert surfaces. PEGylated ShK[Q16K] prevented adoptive-transfer experimental autoimmune encephalomyelitis in rats, a model for multiple sclerosis.

● **Conjugation of ShK to larger proteins:** The circulating half-life of peptides can be prolonged by coupling them to larger proteins or protein domains. By screening a combinatorial ShK peptide library, novel analogues were identified, which when fused to the C-termini of IgG1-Fc retained picomolar potency, effectively suppressed *in vivo* delayed type hypersensitivity and exhibited a prolonged circulating half-life.

● **Prolonged effects despite rapid plasma clearance:** SPECT/CT imaging studies with a 111In-DOTA-conjugate of ShK-186 in rats and squirrel monkeys revealed a slow release from the injection site and blood levels above the channel blocking dose for 2 and 7 days,
respectively. Studies on human peripheral blood T cells showed that a brief exposure to ShK-186 was sufficient to suppress cytokine responses. These findings suggest that ShK-186, despite its short circulating half-life, may have a prolonged therapeutic effect. In rats, the peptide is effective in treating disease in animal models of autoimmune diseases when administered once a day to once in 3 days. In humans, subcutaneous injections twice a week are sufficient to ameliorate disease in patients with plaque psoriasis.

Peptide delivery

The low molecular mass of ShK and its analogues, combined with their high isoelectric points, makes it unlikely that these peptides will be absorbed from the stomach or intestine following oral administration. Sub-lingual delivery is a possibility. A fluorescent ShK analogue was absorbed into the blood stream at pharmacological concentrations following sublingual administration with a mucoadhesive chitosan-based gel, with or without the penetration enhancer cetrimide. Delivery of the peptide as an aerosol through the lung, or across the skin, or as eye drops are also possibilities.

Modulation of T cell function

During T cell activation, calcium enters lymphocytes through store-operated CRAC channels (calcium release activated channel) formed as a complex of Orai and Stim proteins. The rise in intracellular calcium initiates a signaling cascade culminating in cytokine production and proliferation. The Kᵥ1.3 K⁺ channel and the calcium-activated KₑCa3.1 K⁺ channel in T cells promote calcium entry into the cytoplasm through CRAC by providing a counterbalancing cation efflux. Blockade of Kᵥ1.3 depolarizes the membrane potential of T cells, suppresses calcium signaling and IL-2 production, but not IL2-receptor expression. Kᵥ1.3 blockers have no effect on activation pathways that are independent of a rise in intracellular calcium (e.g. anti-CD28, IL-2). Expression of the Kᵥ1.3 and KₑCa3.1 channels varies during T cell activation and differentiation into memory T cells. When naïve T cells and central memory T cells (T₇CM) are activated they upregulate KₑCa3.1 expression to ~500 per cell without significant change in Kᵥ1.3 numbers. In contrast, when terminally differentiated effector memory subsets (TₑM, TₑMRA [T effector memory re-expressing CD45RA]) are activated, they upregulate Kᵥ1.3 to 1500 per cell without changes in KₑCa3.1. The Kᵥ1.3 channel number increases and the KₑCa3.1 channel number decreases as T cells are
chronically activated. As a result of this differential expression, blockers of K\textsubscript{Ca}3.1 channels preferentially suppress the function of naïve and T\textsubscript{CM} cells, while ShK and its analogues that selectively inhibit K\textsubscript{v}1.3 channels preferentially suppress the function of chronically-activated effector memory T cells (T\textsubscript{EM}, T\textsubscript{EMRA}). Of special interest are the large number of ShK analogues developed at Amgen that suppressed interleukin-2 and interferon gamma production by T cells. This inhibitory effect of K\textsubscript{v}1.3 blockers is partial and stimulation strength dependent, with reduced inhibitory efficacy on T cells under strengthened anti-CD3/CD28 stimulation. Chronic-activated CD28\textsuperscript{null} effector memory T cells are implicated in autoimmune diseases (e.g. lupus, Crohn’s disease, rheumatoid arthritis, multiple sclerosis). Blockade of K\textsubscript{v}1.3 channels in these chronically-activated T cells suppresses calcium signaling, cytokine production (interferon gamma, interleukin-2, interleukin 17), and cell proliferation. Effector memory T cells that are CD28\textsuperscript{*} are refractory to suppression by K\textsubscript{v}1.3 blockers when they are co-stimulated by anti-CD3 and anti-CD28 antibodies, but are sensitive to suppression when stimulated by anti-CD3 antibodies alone. In vivo, ShK-186 paralyzes effector-memory T cells at the site of an inflammatory delayed type hypersensitivity response and prevents these T cells from activating in the inflamed tissue. In contrast, ShK-186 does not affect the homing and motility of naïve and T\textsubscript{CM} cells to and within lymph nodes, most likely because these cells express the K\textsubscript{Ca}3.1 channel and are therefore protected from the effect of K\textsubscript{v}1.3 blockade.

**Figure 6 here**

**ShK effects on microglia**

K\textsubscript{v}1.3 plays an important role in microglial activation. ShK-223, an analogue of ShK-186, decreased lipopolysaccharide (LPS) induced focal adhesion formation by microglia, reversed LPS-induced inhibition of microglial migration, and inhibited LPS-induced upregulation of EH domain containing protein 1 (EHD1), a protein involved in microglia trafficking. Increased K\textsubscript{v}1.3 expression was reported in microglia in Alzheimer plaques. K\textsubscript{v}1.3 inhibitors may have use in the management of Alzheimer’s disease, as reported in a recent proof-of-concept study in which a small molecule K\textsubscript{v}1.3 blocker (PAP-1) alleviated Alzheimer’s disease-like characteristics in a mouse model of AD.

**Efficacy of ShK analogues in animal models of human diseases**
- **Experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis:** ShK, ShK-Dap\textsuperscript{22}, ShK-170 and PEGylated ShK-Q16K prevent adoptive-transfer EAE in Lewis rats, a model of multiple sclerosis.\textsuperscript{15,20,40} Since multiple sclerosis is a relapsing-remitting disease, ShK-186 and ShK-192 were evaluated in a relapsing-remitting EAE model in DA (Dark Agouti) rats. Both prevented and treated disease when administered once a day to once in three days.\textsuperscript{(23,31,32)} Thus, Kv1.3 inhibitors are effective in treating disease in rat models of multiple sclerosis when administered alone,\textsuperscript{15,20,23,31,32,40} and therapeutic effectiveness does not appear to be compromised by compensatory over-expression of KCa3.1 channels.\textsuperscript{(67,79)}

- **Pristane-induced arthritis (PIA), a model for rheumatoid arthritis:** ShK-186 was effective in treating PIA when administered every day or on alternate days.\textsuperscript{(21,31)} A scorpion toxin inhibitor of Kv1.3 was also effective in this model.\textsuperscript{(80)} In both these studies, blockade of Kv1.3 alone was sufficient to ameliorate disease and simultaneous blockade of KCa3.1 was not necessary as has been suggested.\textsuperscript{(67,79)}

- **Rat models of atopic dermatitis:** Most infiltrating T-cells in skin lesions from patients with moderate-to-severe atopic dermatitis (AD) express high levels of Kv1.3, suggesting that inhibitors of Kv1.3 may be effective in treating AD.\textsuperscript{(81)} Ovalbumin-induced delayed type hypersensitivity and oxazolone-induced dermatitis are considered to be models of atopic dermatitis.\textsuperscript{(81-83)} ShK, ShK-170, ShK-186, ShK-192, ShK-IgG-Fc and other Kv1.3 inhibitors were all effective in the ovalbumin-induced delayed type hypersensitivity model\textsuperscript{(21,23,24,25,32,33,39,41,46,84-89)}, while a topical formulation of ShK-198 was effective in treating oxazolone-induced dermatitis.\textsuperscript{(81)} Even where compensation by KCa3.1 channels was reported to over-ride Kv1.3 block, ShK administered alone suppressed delayed type hypersensitivity significantly in 2 of 3 studies, albeit modestly.\textsuperscript{(67)}

- **Psoriasis:** Psoriasis is a severe autoimmune disease of the skin that afflicts many people worldwide. Despite the success of recent biologics in ameliorating disease, there is still a search for safe and effective drugs for psoriasis. Kv1.3 inhibitors (ShK, PAP-1) have been reported to treat disease in psoriasiform (psoriasis-like) SCID (severe combined immunodeficiency) mouse models.\textsuperscript{(30,90)} In a recently published Phase 1b placebo-controlled clinical study in patients with plaque psoriasis, ShK-186 administered twice a week (30 or 60 μg/dose/patient) by subcutaneous injection caused improvements with a statistically significant reduction in their PASI (Psoriasis Area and Severity Index) score between
baseline and day 32.(45) These patients also exhibited reduced plasma levels of multiple inflammation markers and decreased expression of T cell activation markers on peripheral blood memory T cells.(45)

- **Diet-induced obesity and fatty liver disease:** Obesity and diabetes are major healthcare problems globally. There is need for safe drugs for these metabolic diseases. In a mouse model of diet-induced obesity, ShK-186 counteracted the negative effects of increased caloric intake. It reduced weight gain, adiposity, and fatty liver; decreased blood levels of cholesterol, sugar, HbA1c, insulin, and leptin; and enhanced peripheral insulin sensitivity.(36) Genetic deletion of the Kv1.3 gene has the same effect, indicating that ShK-186’s effect is due to Kv1.3 blockade.(36,91-93) At least two mechanisms contribute to ShK-186’s therapeutic benefits. The high calorie diet induced Kv1.3 expression in brown fat tissues.(36) By blocking Kv1.3, ShK-186 doubled glucose uptake and increased β-oxidation of fatty acids, glycolysis, fatty acid synthesis and uncoupling protein 1 expression by brown fat.(36) As a consequence of brown fat activation, oxygen consumption and energy expenditure were augmented.(36) The obesity diet also induced Kv1.3 expression in the liver, and ShK-186 caused profound alterations in energy and lipid metabolism in the liver. ShK, its analogues or other Kv1.3 blockers may have use in controlling the negative consequences of high calorie diets.

- **Arousal and anesthesia:** The mechanisms of general anesthesia involve multiple molecular targets and pathways that are not completely understood.(37) Sevoflurane is a common anesthetic used to induce general anesthesia during surgery.(37) Rats continually exposed to sevoflurane lose their righting reflex as an index of loss of consciousness. In these rats, microinfusion of ShK into the central medial thalamic nucleus (CMT) reversed sevoflurane-induced anesthesia in rodents.(37) ShK-treated rats righted themselves fully (restored consciousness) despite being continually exposed to sevoflurane.(37) ShK-microinfusion into neighboring regions of the brain did not have this effect.(37) Sevoflurane enhanced potassium currents in the CMT, while ShK and ShK-186 countered this effect.(37) These studies suggest that ShK-sensitive K+ channels in the CMT are important for suppressing arousal during anesthesia.

- **Preventing brain damage following therapeutic brain radiation:** Brain radiation is used to treat tumors of the head, neck, and brain, but this treatment carries a significant risk of neurologic injury. Injury is, in part, due to the activation of microglia and microglia-mediated damage of neurons. Neuroprotective therapies for radiation-induced brain injury are still
limited. In a mouse model of brain radiation, ShK-170 reversed neurological deficits, and protected neurons from radiation-induced brain injury by suppressing microglia.\(^{(38)}\)

**Toxicity of ShK and its analogues**

- **ShK and ShK-Dap\(^{(22)}\):** ShK peptide has a low toxicity profile in mice. ShK is effective in treating autoimmune diseases at 10 to 100 \(\mu g/kg\) bodyweight. It has a median paralytic dose of approximately 25 mg/kg bodyweight (250-2500 higher than the pharmacological dose). In rats the therapeutic safety index is greater than 75-fold. ShK-Dap\(^{(22)}\) displayed a lower toxicity profile.\(^{(10)}\) A 1.0 mg dose did not induce any hyperactivity, seizures or mortality in rats. The median paralytic dose for ShK-Dap\(^{(22)}\) is about 200 mg/kg bodyweight (2000-20000 higher than pharmacological dose).\(^{(10)}\) PEGylated ShK[Q16K] showed no adverse toxicity in monkeys over a period of several months.\(^{(40)}\)

- **ShK-186/Dalazatide:** ShK-186 also displays a low toxicity profile in rats. Daily administration of ShK-170 or ShK-186 (100 \(\mu g/kg/day\)) by subcutaneous injection over 4 weeks in rats does not induce any changes in blood counts, blood chemistry or histopathology.\(^{(20,21,31)}\) By virtue of suppressing only Tem and TemRA cells, ShK-186 did not compromise protective immune responses to influenza virus and chlamydial infection in rats, most likely because naïve and Tem cells unaffected by Kv1.3 blockade mounted effective immune responses.\(^{(23)}\) ShK-186 is poorly immunogenic and did not elicit anti-ShK antibodies in rats repeatedly administered the peptide.\(^{(21)}\) This is possibly because the peptide’s disulfide-bonded structure hinders processing and antigen presentation by antigen-presenting cells. ShK-186 also shares sequence and structural similarity to a ShK-like domain in matrix metalloprotease 23,\(^{(25-29)}\) which may cause the immune system to assume it is a normal protein in the body. ShK-186 was safe in non-human primates. In Phase 1a and 1b trials in healthy human volunteers, ShK-186 was well tolerated, no grade 3 or 4 adverse effects or laboratory abnormalities were noted, and the predicted range of drug exposures were achieved.\(^{(45)}\) The most common adverse events were temporary mild (Grade 1) hypoesthesis and paresthesia involving the hands, feet, or perioral area. Mild muscle spasms, sensitivity of teeth, and injection site pain were also observed.\(^{(45)}\)

**Functions of ShK-like domains in proteins**

- **MMP-23 and its ShK domain:** MMP-23 belongs to the family of zinc- and calcium-dependent matrix metalloproteases. It is anchored in the cell membrane by an N-terminal
prodomain, and it contains three extracellular domains: catalytic metalloprotease domain, ShK domain and immunoglobulin-like cell adhesion molecule (Ig-CaM) domain.\(^{(25-29)}\) The prodomain traps the voltage-gated potassium channel \(K_v1.3\), but not the closely related \(K_v1.2\) channel, in the endoplasmic reticulum.\(^{(25-29)}\) Studies with chimeras suggest that the prodomain interacts with the \(K_v1.3\) region from the S5 transmembrane segment to the C terminus.\(^{(24-28)}\) NMR studies of the prodomain reveal a single trans-membrane alpha-helix, joined by a short linker to a juxta-membrane alpha-helix, which is associated with the surface of the membrane.\(^{(25-29)}\) The prodomain shares topological similarity with proteins (KCNE1, KCNE2, KCNE4) known to trap potassium channels in the secretory pathway, suggesting a shared mechanism of channel regulation.\(^{(25-29)}\) MMP-23’s catalytic domain displays structural homology with catalytic domains in other metalloproteases, and likely functions as an endopeptidase. MMP-23’s ShK domain lies immediately after the catalytic domain and is connected to the IgCAM domain by a short proline-rich linker. It shares phylogenetic relatedness to sea anemone toxins and ICR-CRISP domains, being most similar to the BgK toxin from sea anemone *Bunodosoma granulifera*.\(^{(25-29)}\) This ShK domain blocks voltage-gated potassium channels (\(K_v1.6 > K_v1.3 > K_v1.1 = K_v3.2 > K_v1.4\), in decreasing potency) in the nanomolar to low micromolar range.\(^{(25)}\) \(K_v1.3\) is required for sustaining calcium signaling during activation of human T cells.\(^{(46,58,59)}\) By trapping \(K_v1.3\) in the endoplasmic reticulum via the prodomain, and by blocking the \(K_v1.3\) channel with the ShK domain, MMP-23 may serve as an immune checkpoint to reduce excessive T cell activation during an immune response. In support, increased expression of MMP-23 in melanoma cancer cells decreases tumor-infiltrating lymphocytes, and is associated with cancer recurrence and shorter periods of progression-free survival.\(^{(28)}\) However, in melanomas, expression of MMP-23 does not correlate with \(K_v1.3\) expression, suggesting that MMP-23’s deleterious effect in melanomas may not be connected with its \(K_v1.3\) channel-modulating function.\(^{(28)}\) MMP-23’s C-terminal IgCAM domain shares sequence similarity with IgCAM domains in proteins known to mediate protein-protein and protein-lipid interactions (e.g. CDON, human Brother of CDO, ROBO1-4, hemicentin, NCAM1 and NCAM2).\(^{(25-29)}\) In summary, the four domains of MMP-23 may work synergistically to modulate immune responses *in vivo*.

- **Mab7, a worm protein containing a ShK domain:** In male *Caenorhabditis elegans* worms, the absence of a protein called Mab7 results in malformed sensory rays that are required for mating.\(^{(94)}\) Introduction of Mab7 into these male worms restores normal development of
normal sensory rays.\textsuperscript{(94)} Introduction of Mab7 proteins lacking the ShK domain does not correct the defect of sensory rays, suggesting a role for the ShK-domain of Mab7 in sensory ray development. \textsuperscript{(94)}

- **HMP2 and PMP1, proteins containing ShK-like domains:** HMP2 and PMP-1 are astacin metalloproteinases from the Cnidarian *Hydra vulgaris* and the jellyfish *Podocoryne carnea* that contain ShK-like domains at their C-termini.\textsuperscript{(29,95)} Both these ShK-domains contain the critical pore-occluding lysine required for K\textsuperscript{+} channel block.\textsuperscript{(25)} HMP2 plays a critical role in foot regeneration of *Hydra*,\textsuperscript{(95)} while PMP1 is found in the feeding organ of the jelly fish and the ShK-domain may paralyze prey after they are ingested.\textsuperscript{(29)}

**Compliance with Ethical Standards**

**Conflicts of Interest**

The author, Shih Chieh Chang, is an inventor of EWSS-ShK. Saumya Bajaj has declared no potential conflicts of interest. K. George Chandy developed ShK-186 and co-authored multiple publications on ShK-186. Kineta Inc. has licensed a patent on ShK-186 from the University of California and is developing this peptide (via Kv1.3 therapeutics) as a therapeutic for autoimmune diseases.

**Human and animal Subjects**

This article does not contain any studies with human or animal subjects.

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Figure 2
Schematic diagram of the primary structure of the ShK peptide highlighting the three disulfide (–S–S–) linkages.
Figure 3

Clockwise from top-left: NMR solution structure of the ShK toxin\(^6\); Two views of the surface representation of ShK highlighting basic (blue) and acidic (red) residues; NMR structures of ShK-192, BmK1, MMP23-ShK, and homology model of EWSS-ShK.
### Table 1

Sequence comparison of ShK with related peptides from other sea anemones, parasitic worms and human MMP23’s ShK domain.

<table>
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<tr>
<th>Toxin Sequence</th>
<th>Toxin Name</th>
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<td>RSCIDTIPR8CTAFU------CRKNSKYRSLF---CRK7CGTC</td>
<td>ShK</td>
<td>E2S063</td>
<td>Stichodactyla helianthus</td>
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<tr>
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<td>Kappa-thalatoxin-Yazza</td>
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<td>Heteractis magnifica</td>
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<td>E2S062</td>
<td>Strongyloides ratti</td>
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<td>E2S061</td>
<td>Stichodactyla haddoni</td>
</tr>
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</tbody>
</table>
Figure 4

Sequence similarity tree between ShK and related peptides generated with NCBI’s Constraint-based Multiple Alignment Tool (COBALT).
Table 2

IC$_{50}$ values for block of potassium channels by ShK and related peptides. ND = not done.

<table>
<thead>
<tr>
<th>Channel</th>
<th>ShK (IC$_{50}$)</th>
<th>ShK-186 (IC$_{50}$)</th>
<th>ShK-192 (IC$_{50}$)</th>
<th>ShK-EWSS (IC$_{50}$)</th>
<th>ShK-F6CA (IC$_{50}$)</th>
<th>ShK-198 (IC$_{50}$)</th>
<th>MMP-23 ShK domain (IC$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_v$1.1</td>
<td>16–28 pM</td>
<td>7 nM</td>
<td>22 nM</td>
<td>5.4 nM</td>
<td>4 nM</td>
<td>159 pM</td>
<td>49 μM</td>
</tr>
<tr>
<td>$K_v$1.2</td>
<td>10 nM</td>
<td>48 nM</td>
<td>ND</td>
<td>&gt;100 nM</td>
<td>&gt;100 nM</td>
<td>ND</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>$K_v$1.3</td>
<td>10–16 pM</td>
<td>70 pM</td>
<td>140 pM</td>
<td>34 pM</td>
<td>48 pM</td>
<td>41 pM</td>
<td>2.8 μM</td>
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<td>$K_v$1.6</td>
<td>200 pM</td>
<td>18 nM</td>
<td>10.6 nM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>400 nM</td>
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<tr>
<td>$K_v$3.2</td>
<td>5 nM</td>
<td>20 nM</td>
<td>4.2 nM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>49 μM</td>
</tr>
<tr>
<td>$K_{Ca}$3.1</td>
<td>30 nM</td>
<td>115 nM</td>
<td>&gt;100 nM</td>
<td>&gt;100 nM</td>
<td>ND</td>
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<td>&gt;100 μM</td>
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</table>
Figure 5

Docking configuration of EWSS-ShK in $K_v1.3$. For clarity, only two of the four $K_v1.3$ subunits in the tetramer are shown. Lys$^{22}$ is shown protruding into the pore and interacting with the channel's selectivity filter.

\[\begin{align*}
W[-3] \\
P377
\end{align*}\]

\[\begin{align*}
&E[-] & & & & & & & & R11 \\
D & G & K22 & & G & D & & & D402 \\
K411 & Y & & G & & Y & & & \\
 & G & & & V & & & V & \\
 & & T & & T & & & & \\
\end{align*}\]
Figure 6

Expression of $K_v1.3$ and $K_{Ca3.1}$ channels during T cell activation and memory T cell generation. The numbers represent functional channel numbers per T cell determined by whole cell patch clamp. The effect of specific blockade of $K_v1.3$ or $K_{Ca3.1}$ on T cell proliferation is shown. $T_{CM} = $ Central memory T cell. $T_{EM} = $ Effector memory T cell. $T_{EMRA} = $ Effector memory T cell that has reacquired CD45RA.