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Analogues of the Sea Anemone Potassium Channel Blocker ShK for the Treatment of Autoimmune Diseases

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Abstract

CCR7⁺ effector memory T (T_{EM}) lymphocytes are involved in autoimmune diseases such as multiple sclerosis, type 1 diabetes mellitus and rheumatoid arthritis. These cells express Kv1.3 potassium channels that play a major role in their activation. Blocking these channels preferentially inhibits the activation of CCR7⁺ T_{EM} cells, with little or no effects on CCR7⁺ naïve and central memory T cells. Blockers of lymphocyte Kv1.3 channels therefore show considerable potential as therapeutics for autoimmune diseases. ShK, a 35-residue polypeptide isolated from the Caribbean sea anemone *Stichodactyla helianthus*, blocks Kv1.3 channels at picomolar concentrations. Although ShK was effective in treating rats with delayed type hypersensitivity and a model of multiple sclerosis, it lacks selectivity for Kv1.3 channels over closely-related Kv1 channels. Extensive mutagenesis studies combined with elucidation of the structure of ShK led to models of ShK docked with the channel. This knowledge was valuable in the development of new ShK analogues with improved selectivity and increasing stability, which have proven efficacious in preventing and/or treating animal models of delayed type hypersensitivity, type 1 diabetes, rheumatoid arthritis, and multiple sclerosis without inducing generalized immunosuppression. They are currently undergoing further evaluation as potential immunomodulators for the treatment of autoimmune diseases.

Keywords

immunomodulator; autoimmunity; venom peptide; Kv1.3 channel; lymphocyte

1. INTRODUCTION

Potassium channels are ubiquitous membrane proteins encoded by 78 genes in the human genome that regulate membrane potential and calcium signaling in diverse cell types [1]. Human T lymphocytes express two types of potassium (K⁺) channels (homotetramers of

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CONFLICT OF INTEREST

All three authors are co-founders of Airmid Inc., a company based in California and focused on the development of Kv1.3 channel blockers as therapeutics for autoimmune diseases.

Kv1.3 and KCa3.1, respectively), the expression of which depends on the state of activation and differentiation of a given T lymphocyte subset [2].

T lymphocytes can be separated into three subsets based on surface expression of the phosphatase CD45RA and the chemokine receptor CCR7 [3]. Mature naïve T lymphocytes express both molecules and therefore are CD45RA⁺CCR7⁺. Central memory T (T_{CM}) lymphocytes, the largest pool of memory T lymphocytes, lose expression of CD45RA during differentiation and therefore are CD45RA⁻CCR7⁺. The third population of T cells is composed of effector memory T (T_{EM}) lymphocytes, which represent fewer than 20% of circulating T lymphocytes and are CD45RA⁻CCR7⁻.

Quiescent human T lymphocytes from all three subsets express 200–300 Kv1.3 and 5–35 KCa3.1 channels per cell [4]. However, activation has diametrically opposite effects on expression of K⁺ channels in the different T lymphocyte populations, leading to an altered channel phenotype. CCR7⁺ naïve and T_{CM} cells up-regulate KCa3.1 channels to ~500/cell, while CCR7⁻ T_{EM} cells increase Kv1.3 expression to 1500 channels/cell with little change in KCa3.1 levels [4]. This switch in channel expression significantly affects responsiveness to Kv1.3 and KCa3.1 blockers, as CCR7⁻ T_{EM} lymphocytes are highly sensitive to Kv1.3 channel blockers while CCR7⁺ naïve/T_{CM} lymphocytes are sensitive to KCa3.1 channel blockers.

Kv1.3 channels play a critical role in the early steps of T-cell activation². Antigen recognition through the T-lymphocyte receptor leads to the rapid release of calcium from endoplasmic reticulum stores. Depletion of these stores causes Ca²⁺ release-activated calcium (CRAC) channels to open in the membrane, and the ensuing calcium influx sustains elevated levels of cytoplasmic calcium. Coordinated activity of calcium-dependent and calcium-independent signaling-pathways culminates in cell activation and proliferation. The large influx of calcium through the CRAC channel induces cell depolarization, which in turn induces a reduction in calcium influx. The driving force for calcium entry is restored by membrane hyperpolarization induced by the efflux of potassium through Kv1.3. and KCa3.1 channels. These potassium channels are therefore necessary for T lymphocyte activation.

The T lymphocytes involved in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and type 1 diabetes mellitus are CCR7⁻ T_{EM} cells, which express large numbers of Kv1.3 channels upon activation [4, 5]. Targeting these CCR7⁻ T_{EM} cells without affecting CCR7⁺ naïve/T_{CM} lymphocytes represents a promising new way of treating these and possibly other autoimmune diseases, without inducing generalized immunosuppression. Moreover, Kv1.3 channels, expressed as homotetramers, have a functionally restricted tissue distribution, and therefore represent attractive therapeutic targets.

Blockers of Kv1.3 and other potassium channels are found in many venoms, including that of sea anemones. In 1995, Castañeda and colleagues extracted a potent K⁺ channel blocker from the Caribbean sea anemone *Stichodactyla helianthus* and named it ShK, for *Stichodactyla helianthus* K⁺ channel toxin [6]. ShK is present in relatively small amounts in *Stichodactyla helianthus* whole body extracts (less than 0.5 mg ShK per kg of extract), limiting the amount of material available for studies of the native peptide. It was, however, synthesized successfully and folded to the biologically active form [7], allowing for extensive studies of its structure, selectivity, and biological activity, and also the generation of analogs with increased selectivity and stability.

2. STRUCTURE, SELECTIVITY, AND *IN VITRO* STABILITY OF SHK AND ITS ANALOGS

2.1. Structure of ShK

ShK is a 35-amino acid peptide with a molecular mass of 4055 Da, and contains 6 cysteine residues located at positions 3, 12, 17, 28, 32, and 35 (Fig. 1) [6, 8]. Its three disulfide bonds were identified as Cys³ to Cys³⁵, Cys¹² to Cys²⁸, and Cys¹⁷ to Cys³² (Fig. 1A) [7, 8].

The alignment of ShK with other potassium channel blockers isolated from sea anemone and scorpion venoms shows that, although they are all of similar length (35–37 amino acids) and all contain three disulfide bonds, potassium channel blockers isolated from sea anemones and scorpions belong to two distinct families (Fig. 1B). The solution structure of ShK [14] is strikingly different from that of scorpion potassium channel blockers. ShK does not contain any β -sheet, but consists of two short α -helices encompassing residues 14–19 and 21–24, and an N-terminus with an extended conformation up to residue 8, followed by a pair of interlocking turns that resembles a 3_{10} -helix (Fig. 2A).

2.2. Interaction of ShK with Kv1.3 Channels

The generation of mono-substituted synthetic analogs of ShK yielded an initial map of the binding site of ShK with human Kv1.3 channels (Fig. 2B) [15]. Amino acid substitutions at the following positions caused no significant effect on the binding of ShK to the channel: Arg¹ (R1S), Phe¹⁵ (F15A, F15W), Lys¹⁸ (K18A), Met²¹ (M21Nle), Tyr²³ (Y23Nph, Y23F, Y23Apa), and Arg²⁴ (R24A). Arg¹¹ is important for the binding of ShK to Kv1.3 as demonstrated by a 42-fold lower affinity of R11Q-ShK for Kv1.3 channels. Lys⁹ plays a significant role in the block of Kv1.3 channels by ShK as K9Q displayed a 9.6-fold lower IC₅₀ than wild-type ShK. Lys²² is a critical residue for the block of Kv1.3 channels by ShK as replacing this residue by an Arg reduced the affinity of the peptide by 225-fold. The IC₅₀ of block of Kv1.3 channels was reduced 23-fold by the mutant K22Nle but only 2-fold by the mutants K22A and K22Orn.

As a next step in identifying the ShK residues involved in peptide-Kv1.3 channel interactions, complementary mutants of ShK and Kv1.3 channels were generated, tested for their affinity by patch-clamp, and analyzed using mutant cycle analysis [16]. Several pairs of significant ShK-Kv1.3 interactions were identified: ShK-Arg¹¹ with Kv1.3-His⁴⁰⁴, ShK-Lys²² with Kv1.3-Tyr⁴⁰⁰, and ShK-Orn²² with Kv1.3-Tyr⁴⁰⁰ and with Kv1.3-Asp⁴⁰².

These findings were later confirmed by alanine scan mutations of ShK [17]. Replacement of Ser²⁰, Lys²², Tyr²³, and Arg²⁴ with Ala residues significantly disrupted the interaction of ShK with Kv1.3 channels. His¹⁹ was replaced by a Lys instead of an Ala because the H19A mutant did not fold in reasonable yield. This H19K mutation significantly disrupted the interaction of ShK with Kv1.3 channels but the role of His¹⁹ may be at least partly structural [18]. Alanine substitutions of residues Arg¹, Thr⁶, Ile⁷, Arg¹¹, Phe¹⁵, Met²¹, Ser²⁶, Phe²⁷, and Arg²⁹ were less disruptive.

Taken together, these data show that ShK binds to the outer vestibule of the Kv1.3 channel pore and that Lys²² protrudes into the pore, thereby blocking the passage for potassium ions. Two ShK amino acid residues (Lys²² and Arg²⁴) play a critical role in Kv1.3 channel blockade by the peptide, three residues (His¹⁹, Ser²⁰, and Tyr²³) have a major role in the ShK-Kv1.3 interaction, six residues (Arg¹¹, Thr¹³, Met²¹, Leu²⁵, Phe²⁷, and Arg²⁹) have an important role, and two residues (Ile⁷ and Ser²⁶) play minor roles. None of the other residues of ShK participates directly in the interaction with Kv1.3 [19].

2.3. Selectivity of ShK for Potassium Channels

The first tests of activity of ShK on potassium channels showed displacement of ^{125}I -dendrotoxin I and ^{125}I - α -dendrotoxin from rat synaptosomal membranes with IC_{50}s of 1 nM and 20 pM, respectively, and inhibition of ^{125}I -charybdotoxin binding to Jurkat T lymphocytes with an IC_{50} of 32 pM. In addition, ShK increased the twitch response of chick biventer cervicis preparations to indirect stimulation, without affecting responses to acetylcholine, carbachol, or KCl, as well as blocking potassium currents in rat dorsal root ganglion neurons, suggesting that it blocked some neuronal potassium channels [6, 7].

Further testing by patch-clamp on cloned potassium channels expressed in mammalian cells showed that ShK blocked Kv1.1 and Kv1.3 channels with IC_{50}s of 16 and 11 pM, respectively. It had a 10-fold lower affinity for Kv1.6 channels and a 30-fold lower affinity for Kv1.4 channels.

ShK blocked Kv1.2, Kv1.7, and KCa3.1 channels in the low nanomolar range, while concentrations of up to 100 nM had no effect on Kv1.5, Kv3.1, and Kv3.4 channels (Table 1) [16]. All blocks were reversible and dose-dependent with Hill coefficients of 1, indicating that one ShK binds per Kv1.3 channel.

$^{86}\text{Rb}^+$ efflux studies showed that ShK blocks Kv3.2 channels with an IC_{50} of 0.6 nM, and electrophysiological studies performed on Kv3.2 channels expressed in *Xenopus laevis* oocytes or in planar patch-clamp studies showed IC_{50} values of 0.3 nM and 6 nM, respectively [26].

2.4. ShK-Dap²², the First ShK Analog with Increased Selectivity for Kv1.3 Over Kv1.1 and Other Potassium Channels

ShK is one of the most potent blockers of Kv1.3 channels. Its main potential drawback as a therapeutic is its lack of selectivity for Kv1.3 over neuronal Kv1.1 channels, as ingress of ShK into the brain could induce neurotoxicity. In order to increase this selectivity, Kalman and colleagues used their knowledge of the structure of ShK and its sites of interaction with Kv1.3 to generate ShK-Dap²² by replacing Lys²² in ShK with the non-protein amino acid diaminopropionic acid (Dap) [16].

The overall structure of ShK-Dap²² is similar to that of wild-type ShK, with both having the same major secondary structure elements [16]. However, ShK-Dap²² has a recognizable helix near its C-terminus (residues 29–32) which was not observed in ShK.

ShK-Dap²² displayed a high affinity for Kv1.3 channels, displacing ^{125}I -charybdotoxin binding to human Kv1.3 channels with an IC_{50} of 102 pM and blocking mouse and human Kv1.3 channels with IC_{50}s of 23 pM (Table 1), and a Hill coefficient close to unity. ShK-Dap²² had a significantly lower affinity than wild-type ShK for Kv1.1, Kv1.2, and Kv1.4 channels, blocked Kv1.6 channels with an IC_{50} in the lower nanomolar range, and had no effect on Kv1.5, Kv1.7, Kv3.1, Kv3.4, or KCa3.1 at concentrations up to 100 nM (Table 1) [16].

2.5. ShK-F6CA, a Fluorophore-Conjugated ShK Analog to Detect Kv1.3-Expressing Cells by Flow Cytometry

Since ShK has such a high affinity for Kv1.3 channels, it was chosen for labeling with a fluorophore in order to detect activated CCR7⁺ T_{EM} lymphocytes that express large numbers of Kv1.3 channels [23]. Based on a detailed knowledge of the structure of ShK and the residues involved in its interaction with Kv1.3, the site chosen for attaching fluorescein was Arg¹, on a region of the peptide facing away from the channel pore. Fluorescein-6-

carboxyl (F6CA) was attached to the α -amino group of ShK-Arg¹ through a 20 Å hydrophilic 2-aminoethoxy-2-ethoxy acetic acid (AEEA; mini-PEG) linker [23].

ShK-F6CA blocked Kv1.3 channels with an IC₅₀ of 48 pM and a Hill coefficient of 1. Intriguingly, ShK-F6CA was 160-fold less effective than wild-type ShK in blocking Kv1.1 channels (IC₅₀ 4 nM for ShK-F6CA and 25 pM for ShK). Moreover, it did not block Kv1.2, Kv1.5, Kv1.7, or Kv3.1 channels at concentrations up to 100 nM (Table 1) [23]. Attaching tetramethylrhodamine (TMR) or biotin to ShK-Arg¹ through an AEEA linker yielded blockers with picomolar affinity for Kv1.3 but no selectivity over Kv1.1 channels. This suggests that the negative charge carried by F6CA play a role in the increased selectivity of ShK-F6CA as TMR is positively charged and biotin neutral.

ShK-F6CA was used to detect Kv1.3 channels on lymphocytes by flow cytometry [21, 23]. Staining intensity paralleled channel numbers measured by whole-cell patch-clamp, with the lower limit of detection using ShK-F6CA being approximately 600 Kv1.3 channels per cell [2, 23]. This detection levels is sufficient to measure the difference in Kv1.3 channels between activated CCR7⁺ naïve/T_{CM} and CCR7⁻ T_{EM} lymphocytes [2, 21, 23].

2.6. ShK-170, ShK-186, and ShK-192, ShK Analogs with Increased Selectivity and Stability

As in the case of ShK-Dap²², this series of ShK analogs was designed to increase ShK's selectivity for Kv1.3 channels over related K⁺ channels, but the strategy chosen for their generation was based on the unexpected selectivity of ShK-F6CA for Kv1.3 (Table 1) [23]. ShK-170 [ShK(L5)] [21], ShK-186 [SL5] [5], and ShK-192 [20] were generated by attaching amino acid derivatives to the N-terminus of ShK through the AEEA linker used previously to attach F6CA to ShK during the synthesis of ShK-F6CA [23].

ShK-170 was generated by attaching ι -phosphotyrosine to ShK *via* the AEEA linker [21]. ShK-170 blocked Kv1.3 channels with an IC₅₀ of 70 pM and was 100-fold selective over Kv1.1 channels, and 700-fold or more selective for Kv1.3 over other K⁺, Na⁺, and Ca²⁺ channels (Table 1) [21]. Attaching δ -phosphotyrosine instead of ι -phosphotyrosine generated a blocker 35-fold selective for Kv1.3 over Kv1.1 channels, but attaching non-phosphorylated Tyr, Phe or Phe derivative yielded blockers with little or no selectivity for Kv1.3 over Kv1.1 channels.

In vitro stability studies showed no evidence of degradation of ShK-170 at ambient temperature (22°C) and pH values ranging from 4.0 to 8.0. At 40°C, ShK-170 was stable at physiological pH but exhibited minor degradation at acidic pH values, mainly due to dephosphorylation of the ι -phosphotyrosine residue attached to Arg¹ through the AEEA linker and oxidation of Met [21]. This instability at non-physiological pH values was more marked at 60°C. ShK-170 contains multiple cleavage sites for trypsin and chymotrypsin, and incubation of the peptide with either or both enzymes lead to rapid degradation of ShK-170 into disulfide-stabilized fragments [20]. This peptide is therefore not amenable to oral delivery without appropriate protection.

ShK-186 is identical to ShK-170, except that its C-terminal Cys was amidated [5]. This modification did not affect the selectivity of the blocker for Kv1.3 over Kv1.1 channels (Table 1), but is known to decrease susceptibility of the C-terminus to carboxypeptidase-mediated cleavage, which may improve the *in vivo* half-life of the peptide [27]. ShK-186 displayed the same *in vitro* stability as ShK-170 at acidic pH and 40°C but was less stable at alkaline pH values [20].

ShK-192 was synthesized to improve peptide stability *in vitro* and *in vivo* [20]. Like ShK-186, ShK-192 was amidated at its C-terminus [5], but, to eliminate oxidation of the

single Met residue of ShK, Met [21] in ShK was replaced with the non-protein amino acid Nle in ShK-192. Finally, to reduce susceptibility to acid-catalyzed hydrolysis of the phosphate from the L-phosphotyrosine residue observed with both ShK-170 and ShK-186, this residue was replaced with the non-hydrolysable phosphate mimetic *para*-phosphonophenylalanine. *In vitro* stability of ShK-192 at ambient temperature was similar to that of ShK-170. At 40°C it was stable at neutral and acidic pH values, but minor degradation was observed at pH 8.0. ShK-192 was stable at 60°C and at acidic pH values and would therefore be suitable for use in slow-release formulations that require such conditions. However, the peptide degraded rapidly at neutral and basic pH when heated to 60°C. NMR spectroscopy studies of ShK-192 showed that it adopts a backbone conformation similar to that of ShK [20]. Differences were noted in the N- and C-termini of ShK-192 and at Nle²¹ and could be ascribed to amidation of the C-terminus of ShK-192, the addition of AEEA-L-phosphotyrosine to its N-terminus, and the replacement of Met²¹ in ShK by Nle. Docking of ShK-192 with the Kv1.3 channel also showed many similarities with ShK docked with Kv1.3 homotetramers [20].

2.7. D-allo-ShK, an ShK Analog Resistant to Endogenous Proteases

D-allo-ShK is a mirror image of native ShK composed entirely of amino acids in the D-configuration at C^α [22]. It was generated because it would be resistant to proteolysis as a result of the inability of endogenous proteases to recognize D-residues. Its immunogenicity was not tested, but it is reasonable to assume that if it could not be processed proteolytically then it would also not be displayed by antigen-presenting cells. D-allo-ShK folded correctly with formation of three disulfide bonds. Its backbone conformation was a mirror image of that of native ShK although chiral side chains such as those of Ile and Thr retained their native configurations.

D-allo-ShK reversibly blocked Kv1.3 channels with a Hill coefficient close to 1 and an IC₅₀ of 36 nM, a value 2,800-fold lower than the affinity of ShK for Kv1.3 channels (Table 1) [22]. Interestingly, D-allo-ShK displayed the same 2-fold selectivity for Kv1.3 over Kv1.1 channels as ShK (IC₅₀ for D-allo-ShK on Kv1.1 channels = 83 nM). A significant difference in the way ShK and D-allo-ShK block Kv1.3 channels resides in the channel conformation they can bind to, with ShK binding and blocking Kv1.3 channels in any conformation (open, closed, and inactivated) but D-allo-ShK not binding to closed channels.

3. IN VITRO BIOLOGICAL ACTIVITY OF SHK AND ITS ANALOGS

The first tests of ShK on lymphocytes showed that it inhibited, with an IC₅₀ in the nanomolar range, the proliferation of peripheral blood human T cells, consisting mainly of naïve and T_{CM} CCR7⁺ cells, stimulated with mitogenic anti-CD3 antibodies (Fig. 3 and Table 2) [4, 16, 22]. In contrast, ShK inhibits the proliferation of human CCR7⁻ T_{EM} cells stimulated with an antigen or a mitogen with IC₅₀ values ranging from 100 to 400 pM [4, 22]. Pre-activated naïve and T_{CM} CCR7⁺ cells can up-regulate KCa3.1 channels even in the presence of ShK, and therefore escape ShK block when reactivated. In contrast, CCR7⁻ T_{EM} cells are unable to up-regulate KCa3.1 channels and therefore remain highly sensitive to ShK [4, 21].

Mouse T lymphocytes exhibit significant differences in their K⁺ channel phenotype when compared to human T lymphocytes and are therefore not useful models for studying Kv1.3 channel blockers [28]. Rat T lymphocytes, however, are very similar to human T lymphocytes in terms of K⁺ channel expression [21, 25, 28]. As with their human counterparts, rat CCR7⁺ naïve and T_{CM} lymphocytes are significantly less sensitive to ShK than rat CCR7⁻ T_{EM} lymphocytes in proliferation assays [21, 25].

ShK-Dap²² inhibited the proliferation of CCR7⁻ T_{EM} lymphocytes with an IC₅₀ of 1.4 nM but had little effect on the proliferation of CCR7⁺ naïve/T_{CM} lymphocytes (Table 2) [16, 25].

ShK-170 was 60-fold more effective in inhibiting the proliferation of CCR7⁻ T_{EM} lymphocytes (IC₅₀ 80 pM) than CCR7⁺ naïve/T_{CM} lymphocytes (IC₅₀ 5 nM) (Fig. 3 and Table 2) [21]. Addition of interleukin-2 (IL-2) partially cancelled the inhibitory effect of ShK-170 on T-lymphocyte proliferation [21], as had been shown with other Kv1.3 blockers [29–31]. ShK-170 (100 nM) competitively inhibited the staining of activated CCR7⁻ T_{EM} cells with ShK-F6CA and inhibited the production of IL-2 by these cells with an IC₅₀ < 1 nM [21]. ShK-170 (100 nM) induced little or no *in vitro* toxicity against human and rat lymphoid cells and was negative in the Ames test on tester strain TA97A, indicating that it is not a mutagen [21].

ShK-186 is to date the best characterized ShK analog in terms of *in vitro* biological activity on CCR7⁺ and CCR7⁻ T lymphocytes (Table 2) [5, 24]. ShK-186 (100 nM) did not affect localization of Kv1.3 channels to the immunological synapse formed between CCR7⁻ T_{EM} lymphocytes and antigen-presenting cells during lymphocyte activation, but it inhibited calcium influx in these cells in a dose-dependent manner with an IC₅₀ of 200 pM [5, 24]. ShK-186 inhibited production of IL-2 and IFN γ by CCR7⁻ T_{EM} lymphocytes more efficiently than in the case of CCR7⁺ naïve/T_{CM} lymphocytes⁵. It was significantly less effective in inhibiting production of IL-4 and TNF α . ShK-186 persistently inhibited the proliferation of CCR7⁻ T_{EM} lymphocytes (IC₅₀ 100 pM) while CCR7⁺ naïve/T_{CM} lymphocytes were 10-fold less sensitive to the blocker at first, and became completely resistant to ShK-186 after their up-regulation of the KCa3.1 channel [5, 24]. ShK-186 also inhibited activation of the adhesion molecule integrin β 1, which plays an important role in the motility of activated CCR7⁻ T_{EM} lymphocytes [24].

ShK-192 inhibited the proliferation of human CCR7⁻ T_{EM} lymphocytes isolated from the synovial fluid of patients with rheumatoid arthritis with an IC₅₀ (200 pM) in the same concentration range as that found with ShK-186 (Table 2) [20].

D-allo-ShK was significantly less potent than ShK in inhibiting CCR7⁻ T_{EM} lymphocyte proliferation (IC₅₀ 10 nM) due to its loss affinity for the channels (Table 2). Like ShK, it was even less potent in inhibiting the proliferation of CCR7⁺ naïve/T_{CM} lymphocytes (IC₅₀ 10 μ M) [22].

4. IN VIVO BIOLOGICAL ACTIVITY OF SHK AND ITS ANALOGS

The species of choice for studying the *in vivo* biological activity of ShK and its analogs is the rat as its T lymphocytes are very similar to their human counterparts in terms of K⁺ channel phenotype and function. In contrast, mouse T lymphocytes do not exhibit this similarity with human T cells [28]. Mice are therefore not relevant for the study of Kv1.3 channel blockers as immunomodulators in studies focused on T lymphocyte-mediated inflammatory diseases [28].

4.1. Pharmacokinetics of ShK and its Analogs in Rats

ShK and ShK-Dap²² were administered intraperitoneally (i.p.) or subcutaneously (s.c.) to rats and their serum concentrations determined by patch-clamp at different time-points [25]. Peak serum concentrations of both peptides (0.1–14 nM) were reached 15 min (i.p.) and 30 min (s.c.) after injection. Their average half-life was approximately 20 min.

In similar studies, ShK-170 was detected in rat serum 5 min after a s.c. injection of 200 µg/kg body weight [21]. Similar to what had been found with ShK and ShK-Dap²², serum peak levels of ShK-170 (12 nM) were reached within 30 min. The half-life was approximately 50 min and levels of ShK-170 reached a baseline of approximately 300 pM over a period of 7 h. Injection of only 10 µg/kg body weight ShK-170 led to a serum peak concentration of 500 pM, a concentration sufficient to block >90% Kv1.3 channels. Repeated daily s.c. injection of 10 µg/kg ShK-170 resulted in levels of 300 pM 24 h after injection.

Rats that received a single s.c. injection of 100 µg/kg body weight of ShK-192 achieved serum levels of 3.5 nM ShK-192 within 30 min. The half-life for this peptide was approximately 30 min but ~200 pM of functionally active ShK-192 was detected 24 and 48 h after injection and ~100 pM could be detected at 72 h [20]. The modifications of ShK-170 used to generate ShK-192 did not lead to an increase in the *in vivo* half-life of the peptide.

D-allo-ShK was administered s.c. to rats (1 mg/kg body weight) and was detectable in the serum 25 min later. Peak levels of 950 nM D-allo-ShK were reached within 50 min of injection and its half-life was estimated to 40 min [22]. This small increase in the half-life of the blocker shows that the major determinant of the short half-life of ShK is not proteolysis but rather elimination through the kidney.

4.2. Therapeutic Efficacy of ShK and its Analogs in Treating Rat Models of Inflammatory Diseases

ShK and ShK-Dap²² were tested in parallel for their efficacy in preventing and treating acute adoptive experimental autoimmune encephalomyelitis (EAE) in rats, an animal model of multiple sclerosis [25]. EAE was induced by the i.p. injection of activated myelin-basic protein specific encephalitogenic T lymphocytes. The *in vitro* pre-treatment of encephalitogenic T cells with 100 nM ShK or ShK-Dap²² prior to i.p. injection, followed by s.c. injections of 80 µg/kg ShK or ShK-Dap²² on the day of cell transfer and for the next 5 days, prevented lethal EAE. In treatment trials, the s.c. injection of 80 µg/kg ShK or ShK-Dap²² for three days, starting on the day of first clinical signs was sufficient to significantly reduce disease severity. ShK, at the same dosage as used for EAE, significantly reduced allergic contact dermatitis induced in rats with oxazolone [32].

ShK-170 was also effective in preventing an active delayed-type hypersensitivity (DTH) reaction and acute adoptive EAE, as well as in treating acute adoptive EAE in rats [21]. Active DTH was induced by immunization of rats with ovalbumin and a subsequent challenge with ovalbumin. A single daily injection of 10 µg/kg body weight ShK-170 after challenge was sufficient to significantly reduce ovalbumin-induced inflammation. ShK-170 was also effective in preventing acute adoptive EAE when administered s.c. once daily (10 µg/kg) for 5 days, starting on the day of encephalitogenic T cell transfer. More importantly, a 3-day treatment after onset of clinical signs also significantly reduced clinical signs of EAE.

ShK-186 (100 µg/kg body weight) prevented active DTH induced against ovalbumin in rats [20]. It also prevented adoptive DTH induced in rats by the i.p. injection of activated ovalbumin-specific CCR7⁻ T_{EM} lymphocytes and subsequent challenge in the ear with ovalbumin, without affecting the number of ear-infiltrating ovalbumin-specific CCR7⁻ T_{EM} lymphocytes [24]. The cells did enter the site of inflammation but two-photon imaging of the tissue showed that they were immotile and were not reactivated by antigen-presenting cells in the presence of ShK-186, at least in part because blocking Kv1.3 channels inhibited activation of the adhesion molecule integrin β1 [24]. The motility of CCR7⁺ naïve/T_{CM} lymphocytes in the lymph node was not affected by ShK-186. This analog at 100 µg/kg body weight showed efficacy in treating two chronic inflammatory diseases, pristane-

induced arthritis, an animal model of rheumatoid arthritis, and chronic-relapsing EAE, an animal model of multiple sclerosis [5, 24]. Arthritis was induced in rats by the s.c. injection of pristane, then rats received ShK-186 or vehicle for 21 days, starting on the day the first clinical sign of arthritis (at least one swollen joint) was observed. ShK-186-treated animals had significantly fewer affected joints than vehicle-treated controls and showed improvement in both radiological and histopathological studies [5]. Chronic-relapsing EAE was induced in rats by a single immunization with rat spinal cord in emulsion with complete Freund's adjuvant. Treatment with ShK-186 (100 µg/kg body weight, administered s.c.) began on the day of the first clinical signs (limp tail) and continued for 40 days. This treatment had no effect on the first episode of disease, when most T lymphocytes infiltrating the central nervous system are CCR7⁺ naïve/T_{CM} cells, but significantly reduced the clinical score of EAE when the disease entered a chronic phase in which the majority of central nervous system-infiltrating T lymphocytes are CCR7⁻ T_{EM} cells [24].

ShK-192, administered s.c. at doses of 1, 10, or 100 µg/kg body weight, significantly reduced inflammation in active DTH induced against ovalbumin in rats [20]. D-allo-ShK, 1 mg/kg body weight administered s.c. at time of challenge with ovalbumin, significantly suppressed an active DTH reaction [22].

4.3. Toxicity Studies of ShK and its Analogs in Mice and Rats

ShK and ShK-Dap²² displayed minimal toxicity in mice, with the median paralytic dose following intravenous injection being 25 mg/kg body weight for ShK and 200 mg/kg body weight for ShK-Dap²² [16].

A major concern when developing K⁺ channel blockers as therapeutics is the potential risk of blocking cardiac Kv11.1 (hERG) channels, thereby inducing potentially fatal arrhythmias. Although ShK-170 did not affect Kv11.1 channels at concentrations up to 100 nM, it was tested in a heart variability assay in conscious rats at 10 µg/kg body weight. No effect was observed on heart rate or on standard heart rate variability parameters in either the time or the frequency domain [21]. Administration of ShK-170 (10 µg/kg/day) for two weeks induced no differences between vehicle and blocker-treated rats in terms of blood counts, blood chemistry, and proportion of thymus and spleen lymphocyte subsets²¹. Injection of a single 1000 µg/kg dose of ShK-170 or of five consecutive daily doses of 600 µg/kg ShK-170 induced no overt toxicity in healthy rats²¹. However, the dose that induces 50% lethality (LD₅₀) was estimated to be 750 µg/kg/day in rats with EAE and a breached blood-brain barrier, probably due to a sufficient blood concentration of ShK-170 (12 nM) to block >50% of neuronal Kv1.1 channels (Table 1) [21].

ShK-186 (100 or 500 µg/kg body weight) was administered s.c. daily for 28 days. No differences between ShK-186-treated and vehicle-treated rats were observed in terms of histopathology, blood counts, or blood chemistry, although irritation at the injection site of ShK-186 was noted [5]. In addition, no toxicity was observed in Rhesus macaques treated once intravenously with 100 µg/kg body weight ShK-186⁵. ShK-186, administered s.c. at a dose that prevented DTH and acute-adoptive EAE and treated acute-adoptive and chronic-relapsing EAE and pristane-induced arthritis (100 µg/kg body weight), did not induce generalized immunosuppression [24]. Rats infected intranasally with either rat-adapted influenza virus or *Chlamydia trachomatis* MoPn were able to clear the pathogens as rapidly as rats treated with vehicle alone. In contrast, rats treated with the broad immunosuppressant dexamethasone displayed a delayed clearance of the virus and were unable to clear the bacteria over the 28 days of the trials.

5. CONCLUSIONS

Millions of people worldwide suffer from autoimmune diseases, which can affect virtually any organ in the body. These chronic disorders are often diagnosed in young adults, or even children, and can be debilitating and lead to premature death. Immunomodulators such as methotrexate, monoclonal antibodies (natalizumab, infliximab), glatiramer acetate, mitoxantrone, TNF antagonists (etanercept, infliximab), and steroids have considerably improved the management of these diseases, but they can cause generalized immunosuppression and therefore an increased risk of tumor cell proliferation or opportunistic infections. Kv1.3 channel blockers, such as ShK and its selective analogs, represent a new class of immunomodulatory compounds with a lower risk of inducing generalized immunosuppression as they preferentially target the CCR7⁻ T_{EM} lymphocytes involved in autoimmune diseases, with little or no effect on CCR7⁺ naïve/T_{CM} lymphocytes.

Findings over the last decade point to a role of Kv1.3 channels in the regulation of peripheral insulin sensitivity and glucose metabolism [33]. In addition to their potential application as immunomodulators, ShK and its analogs may therefore prove effective for the treatment of insulin resistance and type 2 diabetes.

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Fig. (1).

(A) Amino acid sequence of ShK showing its three disulfide bonds. (B) Alignment of potassium channel-blocking peptides isolated from sea anemone and scorpion venoms. AsK: kaliseptine from *Anemonia sulcata* [9]; BgK: *Bunodosoma granulifera* potassium channel toxin [10]; ChTX: charybdotoxin from *Leiurus quinquestriatus*; KTX: kaliotoxin from *Androctonus mauretanicus* [11]; MgTX: margatoxin from *Centruroides margaritatus* [12]; NTX: noxiustoxin from *Centruroides noxius* [13]. Conserved cysteine residues are shown in red (sea anemone peptides) and in blue (scorpion peptides).

Fig. (2).

Structure of ShK toxin. **(A)** Stereo view of solution structure determined by NMR (PDB id 1ROO); disulfide bonds are shown in yellow. **(B)** Surface representation of ShK structure. Residues that contribute to Kv1.3 binding are colored as follows: Lys²² blue, Tyr²³ magenta, Arg²⁴ dark blue, His¹⁹ salmon, Ser²⁰ deep red, and Arg¹¹, Thr¹³, Met²¹, Leu²⁵, Phe²⁷, and Arg²⁹ light green. The right hand view is related to the left hand view by a 90° rotation around the x-axis and a 180° rotation about the y-axis.

A. Human CCR7⁻ T_{EM} cells **B. Human CCR7⁺ naïve and T_{CM} cells**

Fig. (3).
Comparison of the effectiveness of ShK and its analogs in inhibiting the proliferation of (A) human CCR7⁻ T_{EM} cells and (B) human CCR7⁺ naïve and T_{CM} cells [5, 20–22].

Table 1

Selectivity of ShK and its Analogs for Cloned Potassium Channels Expressed in Mammalian Cells. All IC₅₀ Values were Determined by Patch-Clamp and are Given in pM. (N.E.): No Effect

Channel	ShK	ShK-Dap ²²	ShK-F6CA	ShK-170	ShK-186	ShK-192	D-allo-ShK	References
Kv1.1	16	1,800	4,000	7,000	6,900	22,000	83,000	[20–23]
Kv1.2	9,000	39,000	100,000 (N.E.)	48,000			10 ⁶ (N.E.)	[16, 21–23]
Kv1.3	11	23	48	70	70	140	36,000	[5, 20–25]
Kv1.4	312	3,700	100,000 (N.E.)	13,700		100,000 (N.E.)	10 ⁶ (N.E.)	[20–23]
Kv1.5	100,000 (N.E.)	100,000 (N.E.)	100,000 (N.E.)	100,000 (N.E.)		100,000 (N.E.)	10 ⁶ (N.E.)	[16, 20–23]
Kv1.6	165,000	10,500		18,000		11,000		[20, 21]
Kv1.7	12,000	100,000 (N.E.)	100,000 (N.E.)	100,000 (N.E.)			10 ⁶ (N.E.)	[16, 21–23]
Kv3.1	100,000 (N.E.)	100,000 (N.E.)	100,000 (N.E.)	100,000 (N.E.)				[16, 21, 23]
Kv3.2	300 – 6,000			20,000		4,000		[20, 21, 26]
Kv3.4	100,000 (N.E.)	100,000 (N.E.)						[16]
Kv11.1				100,000 (N.E.)		100,000 (N.E.)		[20, 21]
KCa1.1				100,000 (N.E.)				[21]
KCa2.1				100,000 (N.E.)				[21]
KCa2.2				100,000 (N.E.)				[21]
KCa2.3				100,000 (N.E.)				[21]
KCa3.1	30,000	100,000 (N.E.)		115,000		100,000 (N.E.)	10 ⁶ (N.E.)	[16, 20–22]

Table 2

Effects of ShK and its Analogs on T Lymphocyte Functions *In Vitro*. IC₅₀ Values are Given Separately for CCR7⁺ and CCR7⁻ T Lymphocytes. N.D.: Not Determined

	ShK	ShK-Dap ²²	ShK-170	ShK-186	ShK-192	D-allo-ShK	References
CCR7⁺ Naïve and T_{CM} Lymphocytes							
Proliferation	90 nM	> 100 nM	5 nM	2 nM	N.D.	10 nM	[5, 21, 22, 25]
Production of IL-2	N.D.	N.D.	N.D.	2 nM	N.D.	N.D.	[5]
Production of IFN γ	N.D.	N.D.	N.D.	8 nM	N.D.	N.D.	[5]
Production of TNF α	N.D.	N.D.	N.D.	20 nM	N.D.	N.D.	[5]
Production of IL-4	N.D.	N.D.	N.D.	>100 nM	N.D.	N.D.	[5]
CCR7⁻ T_{EM} Lymphocytes							
Calcium influx	N.D.	N.D.	N.D.	200 pM	N.D.	N.D.	[5]
Proliferation	80–400 pM	1.4 nM	80 pM	100 pM	200 pM	10 nM	[5, 20–22, 24, 25]
Production of IL-2	N.D.	N.D.	<1 nM	<100 pM	N.D.	N.D.	[5, 21]
Production of IFN γ	N.D.	N.D.	N.D.	<100 pM	N.D.	N.D.	[5]
Production of TNF α	N.D.	N.D.	N.D.	5 nM	N.D.	N.D.	[5]
Production of IL-4	N.D.	N.D.	N.D.	1 nM	N.D.	N.D.	[5]
b1 integrin activation	N.D.	N.D.	N.D.	<100 nM	N.D.	N.D.	[24]

Table 3

In Vivo Efficacy of ShK and its Analogs in Rat Models of Inflammatory Diseases. All Peptides were Administered s.c. N.D.: Not Determined

Model	ShK	ShK-Dap ²²	ShK-170	ShK-186	ShK-192	p-allo-ShK	References
Prevention of active DTH	N.D.	N.D.	10 µg/kg once daily	100 µg/kg once daily	1–10 µg/kg once daily	1 mg/kg once	[5, 20–22]
Prevention of adoptive DTH	N.D.	N.D.	N.D.	100 µg/kg once daily	N.D.	N.D.	[24]
Prevention of allergic contact dermatitis	16 µg/kg	N.D.	N.D.	N.D.	N.D.	N.D.	[32]
Prevention and treatment of acute adoptive EAE	80 µg/kg 2–4 times daily for 5 days	80 µg/kg 2–4 times daily for 5 days	10 µg/kg once daily for 5 days	N.D.	N.D.	N.D.	[21, 25]
Treatment of chronic-relapsing EAE	N.D.	N.D.	N.D.	100 µg/kg once daily	N.D.	N.D.	[24]
Treatment of pristane-induced arthritis	.	.	.	g/kg once daily	.	.	