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K_v1.3 Selective Peptides Based upon *N*-Terminal Extension and Internal Substitutions of ShK Toxin

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Introduction

With more than 80 different types of autoimmune disorders affecting all organ systems in the human body, finding a drug which would treat patients at the root cause of these diseases has been a quest of our mutual labs for the past two decades. These diseases may range from mild skin disorders such as psoriasis, widely distributed joint damage such as in rheumatoid arthritis, destruction of specific cells such as β cells in type-1 diabetes to more complex disorders affecting central nervous system such as multiple sclerosis. Current treatments involve the use of broad immunosuppressants, which may open the door to opportunistic infections. Through work pioneered with the Chandy lab, we have shown that the channel phenotypes of these autoantigen specific T-cells have preferentially upregulated the K_v1.3 channel to balance Ca^{+2} influx upon activation. A sea anemone derived peptide named ShK is one of the most potent K_v1.3 blockers described (IC_{50} = 10 pM), however it lacks specificity and blocks K_v1.1 and K_v1.5 also at pM levels [1,2]. Through many years of engineering, we have progressed one of our peptides, Dalazatide (formerly ShK-186) to the clinic for its selective block of K_v1.3 channels as a means of treating autoimmune diseases. In our current work, we have built upon our findings to continue to improve the selectivity of ShK-derived analogs and have recently reported selectivity profiles of more than 1000x for K_v1.3 versus K_v1.1. We have improved the drugability of ShK [3,4].

Results and Discussion

Several analogs were designed to investigate the effect of $Q_{16}K$ or $K_{18}A$ substitution on the selectivity and potency of ShK analogs previous reported, such as ShK-192 and ShK analog with *N*-terminal extension EWSS (Figure 1) [3]. The analogs were designed to include a substitution that a group at Amgen had published in a patent filed in 2007. This substitution replaced Gln_{16} with Lys. The results presented in that patent suggested that this substitution conferred on ShK a high K_v1.3 *versus* K_v1.1 specificity. Thus, we incorporated this substitution into ShK-192, which incorporated a non-hydrolyzable para-phosphono-Phe (Ppa) as the *N*-terminal residue, extended from the primary ShK sequence with an Aeea (aminoethyloxyethyloxyacetyl, mini-Peg™) linker, as well as amidation at the *C*-terminus (ShK-224). We also incorporated a Met_{21} to Nle substitution into this sequence to generate an analog that would be less susceptible to oxidation (ShK-223). We also designed two more analogs by replacing the *N*-terminal extension of ShK-192 (Ppa-Aeea) with EWSS extension [3] and replacing Qln_{16} with K (ShK-237), and Lys_{18} with Ala (ShK-238). The EWSS extension consists of only common proteins amino acids, and it was designed to resemble the structure of the ShK-192 *N*-terminal extension [3].

All the analogs were synthesized on a Rink-mBHA resin using an Fmoc-tBu strategy, except for ShK-239 that was synthesized on a Wang resin. The solid-phase assembly proceeded smoothly with all couplings mediated by 6-Cl-HOBT and diisopropylcarbodiimide. In order to form the disulfide bonds utilizing a simple redox buffer, all Cys residues were protected with the trityl group. Following synthesis of the primary chain, each peptide was cleaved from the resin and simultaneously deprotected using an acidolytic reagent cocktail containing carbocation scavengers. The peptides were folded to the active form using a slightly basic aqueous buffer containing an equimolar ratio of reduced and oxidized glutathione. The peptide folding was done for a period of 16 h and was determined to be complete with the formation of the major front eluting peak consistent with other ShK peptides. Each peptide was purified by RP-HPLC and the purified peptides were characterized by analytical HPLC and ESI-MS.

Peptide	Sequence
ShK	H-RSCIDTIPKSRCTQFKCKHSMKYRLSFCRKTCGTC-OH
ShK-013	H-RSCIDTIPKSRCTQFACKHSMKYRLSFCRKTCGTC-NH_2
ShK-192	H-Ppa-Aeea-RSCIDTIPKSRCTQFQCKHSNleKYRLSFCRKTCGTC-NH_2
ShK-223	H-Ppa-Aeea-RSCIDTIPKSRCTQF**K**CKHSNleKYRLSFCRKTCGTC-NH_2
ShK-224	H-Ppa-Aeea-RSCIDTIPKSRCTQF**K**CKHSMKYRLSFCRKTCGTC-NH_2
ShK-237	H-**EWSS**-RSCIDTIPKSRCTKF**K**CKHSMKYRLSFCRKTCGTC-NH_2
ShK-238	H-**EWSS**-RSCIDTIPKSRCTQFQC**A**HSMKYRLSFCRKTCGTC-NH_2
ShK-239	H-**EWSS**-RSCIDTIPKSRCTQFKCKHSMKYRLSFCRKTCGTC-OH

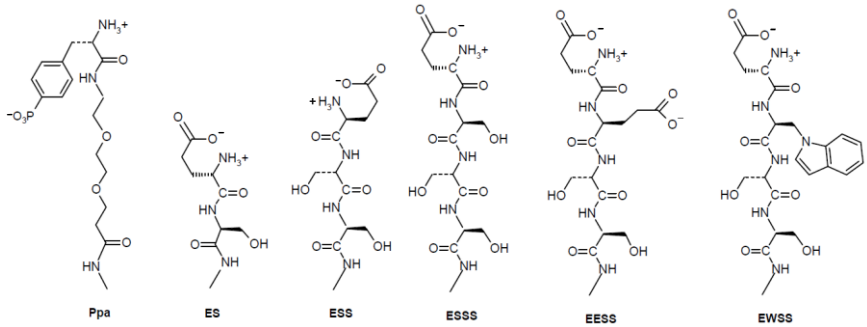

Fig. 1. N-terminal extensions of ShK analogues. Schematic illustrating the structural similarity and physicochemical properties of Ppa moiety of ShK-192 and various amino acid extensions.

We used patch-clamp electrophysiology to assess the effects of ShK-223, ShK-224, ShK-237, ShK-238 and ShK-239 on $K_v1.1$ and $K_v1.3$ channels and compare them to the parent peptide, ShK and ShK-192. Mouse fibroblasts stably expressing homotetramers of $K_v1.1$ or $K_v1.3$ were patch-clamped in the whole-cell configuration and steady-state block was measured after addition of different concentrations of the peptides. As published previously, ShK inhibited $K_v1.3$ currents with an IC_{50} of 10 pM and $K_v1.1$ currents with an IC_{50} of 25 pM (Table 1). ShK-224 exhibited a 16-fold loss of efficacy on $K_v1.3$ relative to ShK, ShK-237 a 26-fold loss, ShK-238 a 9-fold loss and ShK-239 a 3-fold loss, whereas the potency of ShK-223 on $K_v1.3$ was similar to that of ShK (Table 1). Unlike ShK, ShK-238 exhibited a 28-fold selectivity for $K_v1.3$ over $K_v1.1$, and ShK-239 a 158-fold selectivity, while ShK-223, ShK-224 and ShK-237 had any effect on $K_v1.1$ currents at concentrations up to 100 nM, representing a selectivity for $K_v1.3$ of >10,000-fold.

Table 1. Comparative IC_{50} Values for $K_v1.3$ and $K_v1.1$.

Peptide	*$K_v1.3$ IC_{50} (pM)*	*$K_v1.1$ IC_{50} (pM)*	*Ratio($K_v1.1/K_v1.3$)*
ShK	10	25	2.5
ShK-013	40	4900	122.5
ShK-192	140	22,000	157
ShK-223	25	>100,000	>4000
ShK-224	164	>100,000	>609
ShK-237	263	>100,000	>380
ShK-238	88	2480	28
ShK-239	34	5372	158

In this study we showed that ShK analogs with *N*-terminal extensions are conveniently produced *via* standard Fmoc/tBu methods. These analogs were modeled to resemble the ShK-192 with the *N*-terminal extension *para*-Phosphono-Phe with an Aeea linker or where an anionic Glu is positioned in close proximity to Trp giving a similar aromatic anionic charge positioned by two spacer Ser residues. Additionally, we have also introduced two previously determined selectivity determinants, either $Q_{16}K$ or $K_{18}A$ [3] coupled with these *N*-terminal additions. The *N*-terminal extension of EWSS confers a similar 100x selectivity of $K_v1.3$ versus $K_v1.1$ as that of ShK-192. The $Q_{16}K$ substitution coupled with either the Ppa-Aeea or EWSS *N*-terminal extensions confers the greatest level of selectivity enhancement as shown in the ShK-223, ShK-224 and ShK-237. The $K_{18}A$ coupled with the EWSS *N*-terminal extension lost more than 10x of its gain of the singly substituted ShK-$K_{18}A$ [5].

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