Kv1.3 Antagonists and Methods of Use

This application claim the benefit of U.S. Provisional Application No. 61/757,389, filed 28 January 2013, and the U.S. Provisional Application No. 61/756,777, filed 25 January 2013, the entire contents of which are incorporated herein by reference.

Field of the Invention

The present invention relates to antagonists of Kv1.3, polynucleotides encoding them, and methods of making and using the foregoing. The antagonists are based on variants of the OdK2 peptide.

Background of the Invention

Ion channels regulate a diversity of cellular functions through generation of ionic currents, including cardiac, CNS, and immune physiology. It is estimated that between 5-30% of marketed drugs may regulate ion channel activity (Overington *et al.*, Nat Reviews Drug Discovery 5:993-6, 2006). Subfamily selectivity is a desired feature of new therapeutics to improve efficacy and safety of current non-selective drugs, and poses a significant challenge for small molecules and known naturally occurring peptide toxins (Wickenden *et al.*, Future Med Chem 4:661-79, 2012). This is especially true within large homologous families such as voltage-gated K^+, Ca^+ and Na^+ channels.

Kv1.3, the potassium voltage-gated channel subfamily A member 3, is expressed on T cells and functions to regulate T cell activation. Sustained calcium signaling is required for T cell activation for upregulation of cell surface activation markers and increase in cytokine production and proliferation via calcineurin dependent dephosphorylation and nuclear translocation of nuclear factor of activated T cells (NFAT). Inositol triphosphate (IP3) dependent release of internal calcium stores from the endoplasmic reticulum activates the

calcium release activated calcium channels (CRAC) on the cell surface, providing an influx of extracellular calcium and sustained calcium signaling (reviewed in Cahalan *et al.*, Immunol Rev 231:59-87, 2009). An efflux of potassium is required for the cells to remain in a hyperpolarized state and for calcium influx to be maintained for full T cell activation. This potassium efflux appears to be regulated through the voltage-gated potassium channel Kv1.3 and the calcium-activated potassium channel KCa3.1. Blockers selective for Kv1.3 have demonstrated that Kv1.3 is the potassium channel responsible for regulating calcium signaling, even in the absence of any inhibition of KCa3.1. (Beeton *et al.*, Mol Pharmacol 67:1369-81, 2005). Blocking Kv1.3 depolarizes T cells and inhibits calcium entry, cytokine production, and proliferation of activated T cells *in vitro* (reviewed in Cahalan *et al.*, Immunol Rev 231:59-87, 2009).

Kv1.3 blockers have been shown to reduce T cell dependent disease progression in autoimmune models, such as experimental autoimmune encephalomyelitis (EAE), experimental arthritis, delayed-type hypersensitivity (DTH), allergic contact dermatitis and glomerulonephritis (Rangaraju *et al.*, Expert Opin Ther Targets 13:909-24, 2009; Beeton *et al.*, Proc Natl Acad Sci U S A. 103:17414-9, 2006; Koo *et al.*, J Immunol 158:5120-8, 1997; Hyodo *et al.*, Am J Physiol Renal Physiol 299:F1258-69, 2010). The calcium calcineurin NFAT pathway inhibitors cyclosporine A (*Neoral*, *Sandimmune*, *Gengraf*) and Tacrolimus (FK-506 or fujimycin) are approved treatments for severe immune disorders, including transplant rejection and severe rheumatoid arthritis. The broad distribution of calcineurin in tissues such as kidneys may result in a higher degree of mechanism based toxicity, narrow safety margins, and limited therapeutic application for these compounds. T cell inhibition using selective Kv1.3 blockers may result in increased safety profile and greater efficacy in the

treatment of T cell mediated inflammatory and autoimmune diseases.

Kv1.3 may play a role in regulating weight gain and improving insulin sensitivity. Kv1.3 deficient mice show reduced weight gain, higher insulin sensitivity, and reduced plasma glucose levels (Xu *et al.*, Hum Mol Genet 12:551-9, 2003). Kv1.3 blockers have been shown to increase glucose transporter 4 (GLUT4) cell surface expression in skeletal muscle and adipose tissue, and result in increased insulin sensitivity in normal and ob/ob obese mice, and to increase glucose uptake in primary adipocytes *in vitro* (Xu *et al.*, Proc Natl Acad Sci USA 101:3112-7, 2004). In humans, a single nucleotide polymorphism (SNP) in the Kv1.3 gene has been associated with decreased insulin sensitivity and impaired glucose tolerance (Tschritter, Clin Endocrinol Metab 91:654-8, 2006).

Kv1.3 may have a critical function in smooth muscle proliferative disorders like restenosis in patients following vascular surgery, such as angioplasty. Kv1.3 expression is increased in proliferating human and mouse smooth muscle cells. Kv1.3 blockers inhibit calcium entry, reduce smooth muscle cell migration, and inhibit neointimal hyperplasia in *ex vivo* human vein samples (Cheong *et al.*, Cardiovasc Res 89:282-9, 2011).

Increasing evidence indicates that Kv1.3 channels are involved in the activation and/or proliferation of many types of cells, including tumor cells (Bielanska *et al.*, Curr Cancer Drug Targets 9:904-14, 2009), microglia (Khanna *et al.*, Am J Physiol Cell Physiol 280:C796-806, 2001) and differentiation of neuronal progenitor cells (Wang *et al.*, J Neurosci 30:5020-7, 2010) suggesting that Kv1.3 blockers may be beneficial in the treatment of neuroinflammatory and neurodegenerative diseases, and cancers.

Toxin peptides produced by a variety of organisms have evolved to target ion channels. Snakes, scorpions, spiders, bees, snails, sea anemone, insects, arachnids, cnidarians,

reptiles, and mollusks are a few examples of organisms that produce venom that can serve as a rich source of small bioactive toxin peptides or "toxins" that potently and selectively target ion channels and receptors. In most cases, these toxin peptides have evolved as potent antagonists or inhibitors of ion channels, by binding to the channel pore and physically blocking the ion conduction pathway or by antagonizing channel function by binding to a region outside the pore (e.g., the voltage sensor domain). Toxin peptides are typically about 20-80 amino acids long with distinct disulfide bond pairing, and can be divided into a number of superfamilies based on their disulfide connections and peptide folds. Many venom toxins are being engineered to improve their properties such as selectivity (King, Expert Opin Biol Ther 11:1469-84, 2011; Escoubas and King, Expert Review Proteomics 6:221-4, 2009).

Venom peptides demonstrating Kv1.3 blocking include ShK, OdK2, OsK1, margatoxin, kaliotoxin etc (see Chandy *et al.*, Trends in Pharmacol Sci 25:280-9, 2004). Kv1.3 blockers OdK2 and OsK1 (alpha-KTx3.7) are homologous members of the α-KTx3 scorpion toxin family from the venom of *Odontobuthus doriae* and *Orthochirus scrobiculosus*, respectively (Abdel-Mottaleb *et al.*, Toxicon 51:1424-30, 2008; Mouhat *et al.*, Biochem J 385(Pt 1):95-104, 2005; Int. Pat. Publ. No. WO2006/002850). OsK1 (alpha-KTx3.7) was reported to block Kv1.3, Kv1.1 and Kv1.2 channels potently and KCa3.1 channel moderately (Mouhat *et al.*, Biochem J 385(Pt 1):95-104, 2005). OdK2 (alpha-KTx3.11) was reported to block Kv1.3 while having no activity on Kv1.1, Kv1.2, Kv1.4, Kv1.5, and Kv1.6) (Abdel-Mottaleb *et al.*, Toxicon 51:1424-30, 2008; Epub 2008 Mar 29).

Engineered toxin peptides with improved potency, selectivity and/or half life including OsK1 and ShK have been reported (Int. Pat. Appl. Publ. WO2006/002850; Int. Pat. Appl. Publ. WO2006/042151; Int. Pat. Appl. Publ. WO2008/088422, Int. Pat. Appl. Publ. WO2006/116156).

There exists a need for more potent and selective Kv1.3 blockers for the therapeutic treatment of Kv1.3-mediated diseases such as T-cell mediated inflammatory and autoimmune diseases such as lupus and multiple sclerosis.

Brief Description of the Drawings

Figure 1 Amino acid sequence alignment of native OdK2 (SEQ ID NO: 1) and OsK1 (SEQ ID NO: 2) (shown as OsK-1 in the figure). Cysteine residues are highlighted in grey. Disulfide bridges and cysteine pairs are shown. The nine divergent residues between OdK2 and OsK1 are highlighted in black.

Figure 2 Binding of A) KV1C2 (Odk2-Fc fusion) and B) KV1N2 (OsK1-Fc fusion) to Kv1.3 E3C cells (solid black), Kv1.3 E3C cells in the presence of a 10-fold excess ShK (dashed grey), and to Kv1.5 (negative control cells; dotted grey). Binding was detected with anti-human Fc-Cy5 using flow cytometry. Data are shown as histogram overlays of Geometric mean fluorescence intensity (GMFI) (Geom. Mean, Red A: Cy5).

Figure 3. Inhibition of memory T cell proliferation by KV1C2 (OdK2-Fc fusion) (▼). Each data point is the mean ± SD of triplicate reactions. Negative control IgG4 Fc (○) did not inhibit T-cell proliferation.

Figure 4. A) Amino acid sequences, B) Activity and selectivity of peptide variant fusion proteins determined in binding and thallium flux assays using cells expressing Kv1.3 and Kv1.1.

Figure 5. A) Inhibition of T cell activation by purified Odk2 chimera Fc fusion proteins at single 100 nM concentration. KV1B03 (■) is identical to KV1C2 (OdK2 Fc fusion) (□). B) Concentration dependent inhibition of T cell activation by KV1D261. Negative control IgG4 Fc did not inhibit T-cell IL-2 production. C) Correlation between binding to Kv1.3 E3C cells and T-cell inhibition for select variants.

Figure 6. Activity of p261 C-terminal extension HSA fusion protein library. Amino acid sequences of the C-terminal extensions and the resulting extended p261 amino acid sequences are shown, along with activity in binding and thallium flux assays.

Figure 7. Characterization of A) p261 B) p579 C-terminal extension HSA fusion proteins.

Figure 8. Characteristics of select OdK2 variant fusion proteins.

Figure 9. Pharmacokinetics of KV1D261_34 in minipigs.

Figure 10. Ex-vivo inhibition of IL-17A secretion from lymphocytes following *in vivo* administration of KV1D261_34 in minipigs.

Figure 11. Cell numbers from draining lymph nodes at day 10 following antigen challenge in the delayed type hypersensitivity (DTH) minipig model.

Summary of the Invention

The invention provides an isolated peptide antagonist of Kv1.3 having an amino acid sequence comprising:

(i) the sequence shown in SEQ ID NO: 1 having a substitution of glycine to isoleucine at position 10 (G10I), and optionally having 1, 2, 3, 4, 5, 6 or 7 additional substitutions; or

(ii) an amino acid sequence which is at least 80% identical to SEQ ID NO: 1, further comprising a G10I substitution.

The invention also provides an isolated peptide antagonist of Kv1.3 comprising the sequence of SEQ ID NO: 55 or 86.

The invention also provides fusion proteins comprising the peptide antagonist of the invention.

The invention also provides an isolated polynucleotide encoding the peptide antagonist or fusion protein of the invention.

The invention also provides a vector comprising the isolated polynucleotide of the invention.

The invention also provides a host cell comprising the vector of the invention.

The invention also provides a method of producing the antagonist or fusion protein of the invention, comprising culturing the host cell of the invention and recovering the antagonist or fusion protein expressed by the host cell.

The invention also provides a pharmaceutical composition comprising the antagonist or fusion protein of the invention and a pharmaceutically acceptable carrier.

The invention also provides a method of suppressing T cell activation in a subject having a condition associated with undesired T cell activation, comprising administering to the subject an effective amount of the antagonist or fusion protein of the invention to suppress T cell activation.

Detailed Description of the Invention

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

As used herein and in the claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which an invention belongs. Although any compositions and methods similar or equivalent to those described herein can be used in the practice or testing of the invention, exemplary compositions and methods are described herein.

The term "polypeptide" means a molecule that comprises at least two amino acid residues linked by a peptide bond to form a polypeptide. Polypeptides of less than about 80 amino acids may be referred to as "peptides". Polypeptides may also be referred as "proteins".

The term "polynucleotide" means a molecule comprising a chain of nucleotides covalently linked by a sugar-phosphate

backbone or other equivalent covalent chemistry. Double and single-stranded DNAs and RNAs are typical examples of polynucleotides.

The term "complementary sequence" means a second isolated polynucleotide sequence that is antiparallel to a first isolated polynucleotide sequence and that comprises nucleotides complementary to the nucleotides in the first polynucleotide sequence.

The term "vector" means a polynucleotide capable of being duplicated within a biological system or that can be moved between such systems. Vector polynucleotides typically contain elements, such as origins of replication, polyadenylation signal or selection markers that function to facilitate the duplication or maintenance of these polynucleotides in a biological system. Examples of such biological systems may include a cell, virus, animal, plant, and reconstituted biological systems utilizing biological components capable of duplicating a vector. The polynucleotides comprising a vector may be DNA or RNA molecules or hybrids of these.

The term "expression vector" means a vector that can be utilized in a biological system or a reconstituted biological system to direct the translation of a polypeptide encoded by a polynucleotide sequence present in the expression vector.

The term "wild type OdK2" or "OdK2" or "native OdK2" as used herein refers to scorpion *Odontobuthus doriae* OdK2 polypeptide having a sequence shown in SEQ ID NO: 1 (GVPTDVKCRGSPQCIQPCKDAGMRFGKCMNGKCHCTPK).

The term "wild type OsK1" or "OsK1" or "native OsK1" as used herein refers to scorpion *Orthochirus scrobiculosus* OsK1 polypeptide having a sequence shown in SEQ ID NO: 2 (GVIINVKCKISRQCLEPCKKAGMRFGKCMNGKCHCTPK).

The term "variant" or "OdK2 variant" as used herein refers to a polypeptide that differs from the wild type OdK2 polypeptide of SEQ ID NO: 1 by one or more modifications for

example, substitutions, insertions or deletions of nucleotides or amino acids.

Throughout the specification, residue numbering of OdK2 variants is according to SEQ ID NO: 1. For example, "G10" in the specification refers to the glycine residue at position 10 of SEQ ID NO: 1. Accordingly, OdK2 G10I refers to an OdK2 variant having glycine at position 10 substituted for isoleucine, and OdK2 G10I,P12R refers to an OdK2 variant having glycine at position 10 substituted for isoleucine, and proline at position 12 substituted for arginine.

Numbering of a given amino acid or polynucleotide sequence "corresponds to" or is "relative to" the numbering of a selected amino acid or polynucleotide sequence when the position of any given amino acid residue or nucleotide residues is designated by reference to the same or to an equivalent position in the selected amino acid or polynucleotide sequence rather than by the actual numerical position of the component in the sequence. Thus, for example, the numbering of a given amino acid position in a given polypeptide sequence corresponds to the same or equivalent amino acid position in a selected polypeptide sequence used as a reference sequence.

An "equivalent position" (for example, an "equivalent amino acid position" or "equivalent nucleic acid position" or "equivalent residue position") is defined herein as a position (such as, an amino acid position or nucleic acid position or residue position) of a test polypeptide (or test polynucleotide) sequence which aligns with a corresponding position of a reference polypeptide (or reference polynucleotide) sequence, when optimally aligned using an alignment algorithm as described herein. The equivalent amino acid position of the test polypeptide need not have the same numerical position number as the corresponding position of the reference polypeptide; likewise, the equivalent nucleic acid position of the test polynucleotide need not

have the same numerical position number as the corresponding position of the reference polynucleotide.

Two polypeptide sequences are "optimally aligned" when they are aligned using defined parameters, i.e., a defined amino acid substitution matrix, gap existence penalty (also termed gap open penalty), and gap extension penalty, so as to arrive at the highest similarity score possible for that pair of sequences. The BLOSUM62 matrix (Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89(22):10915-10919) is often used as a default scoring substitution matrix in polypeptide sequence alignment algorithms (such as BLASTP). The gap existence penalty is imposed for the introduction of a single amino acid gap in one of the aligned sequences, and the gap extension penalty is imposed for each residue position in the gap. Unless otherwise stated, alignment parameters employed herein are: BLOSUM62 scoring matrix, gap existence penalty = 11, and gap extension penalty = 1. The alignment score is defined by the amino acid positions of each sequence at which the alignment begins and ends (e.g. the alignment window), and optionally by the insertion of a gap or multiple gaps into one or both sequences, so as to arrive at the highest possible similarity score.

"Kv1.3" (also known as KCNA3, HPCN3, HGK5, HuKIII, or HLK3) as used herein refers to the well known human potassium voltage-gated channel subfamily A member 3 having a sequence shown in UniProt accession number P22001 and in SEQ ID NO: 418.

"Antagonist of Kv1.3" or "antagonist" as used herein refers to an OdK2 variant or OdK2 variant fusion protein of the invention that inhibits or blocks Kv1.3 function by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 100%. Amino acid sequence of the wild type OdK2 is shown in SEQ ID NO: 1.

"Fusion protein" as used herein refers to a protein that includes polypeptide or peptide components derived from more than one parental polypeptide or peptide.

"Half-life extending moiety" as used herein refers to a molecule or protein or domain that, when conjugated to the OdK2 variant increases the *in vivo* half life of the resulting OdK2 variant fusion protein when compared to the free peptide.

"Percent binding" or "%Binding" as used herein refers to a ratio of geometric mean fluorescence intensities (Geo. MFI or GMFI) for an OdK2 variant fusion protein when compared to the control, obtained from a FACS assay using cells expressing Kv1.3 or Kv1.1 channels.

"Binding selectivity" as used herein refers to the ratio of %Binding obtained for Kv1.3 to %Binding obtained for Kv1.1.

"Selective" or "selectivity" as used herein refers to the ratio of an IC_{50} value for Kv1.1 to an IC_{50} value for Kv1.3 for an OdK2 variant fusion protein or OdK2 variant. Selectivity can be assessed using various methodologies, for example electrophysiological patch clamp assays or thallium flux assays as described herein. Selectivity may vary slightly depending on the assay chosen for measurements.

The Kv1.3 blocking peptides Odk2 (SEQ ID NO: 1) and Osk1 (SEQ ID NO: 2) are members of the α-KTx3 scorpion toxin family that differ in amino acid sequence at nine positions. Both OdK2 and Osk1 are 38 amino acids in length, and are each stabilized by three disulfide bonds with paring between Cys8-Cys28, Cys14-Cys33, and Cys18-Cys35 (Abdel-Mottaleb *et al.*, Toxicon 51:1424-30, 2008; Mouhat *et al.*, Biochem J. 385(Pt 1):95-104, 2005; Int. Pat. Publ. No. WO2006/002850). The folded peptides form an α-helix held in close proximity to a 3 stranded anti parallel β-sheet by the disulfide bonds. OdK2 and OsK1 are pore blockers that inhibit channel function through binding to the outer vestibule of the pore region, inserting lysine 27 into the water filled pore, and occluding ion flow. OsK1 (alpha-KTx3.7) is reported to block Kv1.3, Kv1.1 and Kv1.2, channels potently and KCa3.1 channel moderately, with an IC_{50} of 0.014 nM, 0.6 nM, 5.4 nM, and 225

nM, respectively (Mouhat *et al.*, Biochem J 385(Pt 1):95-104, 2005). OdK2 (alpha-KTx3.11) is reported to block Kv1.3 in Xenopus laevis oocytes, with an IC_{50} of 7.2 nM, and is reported to have no activity on other Kv1.x subtypes tested (Kv1.1, Kv1.2, Kv1.4, Kv1.5, and Kv1.6) (Abdel-Mottaleb *et al.*, Toxicon 51:1424-30, 2008). These data indicate that OsK1 is very potent but lacks sufficient subtype selectivity, whereas OdK2 appears selective but not highly potent.

The present invention provides isolated OdK2 variants and OdK2 variant fusion proteins that inhibit Kv1.3, polynucleotides encoding them, vectors, host cells, and methods of using the polynucleotides and polypeptides of the invention. The OdK2 variants and OdK2 variant fusion proteins of the invention are more potent towards Kv1.3 when compared to the parent molecules with retained and/or enhanced selectivity. The polypeptides of the invention inhibit potassium currents, thallium flux and/or T cell activation resulting from Kv1.3 activity and therefore may be useful in the treatment of various conditions associated with activated T cells, such as inflammatory and autoimmune diseases.

Antagonist Peptides

The invention provides an isolated peptide antagonist of Kv1.3 having an amino acid sequence comprising:

(i) the sequence shown in SEQ ID NO: 1 having a substitution of glycine to isoleucine at position 10 (G10I), and optionally having 1, 2, 3, 4, 5, 6 or 7 additional substitutions; or

(ii) an amino acid sequence which is at least 80% identical to SEQ ID NO: 1, further comprising a G10I substitution.

In some embodiments the isolated peptide antagonist comprises a sequence with no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, or no more than 2 substitutions relative to SEQ ID NO: 1.

In some embodiments the isolated peptide antagonist comprises a sequence with at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 1.

In some embodiments, therefore, the peptide antagonist of Kv1.3 may comprise the sequence of any one of SEQ ID NOs: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, or 110. The substitution G10I is associated with improved selectivity and/or improved affinity for Kv1.3.

In some embodiments described herein, the antagonist of Kv1.3 comprises the sequence GVPXaa$_1$Xaa$_2$VKCXaa$_3$ISRQCXaa$_4$Xaa$_5$PCKDAGMRFGKCMNGKCHCTPK (SEQ ID NO: 426); wherein

a) Xaa1 is I or T, Q or E;

b) Xaa2 is N or D;

c) Xaa3 is K or R, E, A or Q;

d) Xaa4 is I, E, L, D, Q, H, V, K or A;

e) Xaa$_5$ is E K, L, Q, D, V or H; and

the peptide antagonist of Kv1.3 has an optional C-terminal extension of four amino acids. For example, the peptide antagonist of Kv1.3 may comprise the amino acid sequence of SEQ ID NOs: 3, 13, 21, 22, 24, 26, 29, 30, 32, 34, 38, 39, 42-46, 49, 51, 59, 63, 65, 69, 71, 73, 76, 78, 81-83, 85, 87, 89, 92, 96, 101, 103, 104 and 108.

In some embodiments described herein, the antagonist of Kv1.3 comprises the sequence

GVPXaa$_1$Xaa$_2$VKCXaa$_3$ISRQCXaa$_4$Xaa$_5$PCKDAGMRFGKCMNGKCHCTPK (SEQ ID NO: 427); wherein

Xaa$_1$ is I or T;

Xaa$_2$ is N or D;

Xaa$_3$ is K or R;

Xaa$_4$ is I or E; and

Xaa$_5$ is E or K; and

the peptide antagonist of Kv1.3 has an optional C-terminal extension of four amino acids. For example, the peptide antagonist of Kv1.3 may comprise the amino acid sequence of SEQ ID NOs: 3, 22, 34 or 42.

As will be seen by reference to figure 4A, the peptide antagonists of the invention retain the native disulfide bridges between C8-C28, C14-C33 and C18-C35. The exemplary antagonists of the invention described in this section and elsewhere can have substantially enhanced selectivity for Kv1.3 against Kv1.1, for example 100, 200, 300, 400, 500, 1000, 2000, 3000, 4000, 5000, 6000 or at least 7000 fold improved selectivity compared to SEQ ID NO: 1.

Fusion proteins

The antagonist of the invention can be an isolated fusion protein that comprises an antagonist peptide of the invention. In some embodiments described herein, the fusion protein comprises a sequence with at least 80% identity to SEQ ID NO: 1, and which comprises the substitution G10I relative to SEQ ID NO: 1.

In some embodiments described herein, the fusion protein comprises a peptide antagonist of Kv1.3 conjugated to a half-life extending moiety, wherein the peptide antagonist of Kv1.3 comprises the sequence shown in SEQ ID NO: 1, further comprising a substitution of G10I, and optionally having 1, 2, 3, 4, 5, 6 or 7 additional substitutions.

In some embodiments described herein, the fusion protein comprises a peptide antagonist of Kv1.3 conjugated to a half-life extending moiety, wherein the peptide antagonist of

Kv1.3 comprises the sequence at least 80% identical to the amino acid sequence shown in SEQ ID NO: 1, further comprising a substitution of glycine to isoleucine at residue position 10 (G10I).

In some embodiments, the fusion protein comprises a peptide antagonist of Kv1.3 conjugated to a half-life extending moiety, wherein the peptide antagonist of Kv1.3 comprises the sequence GVPXaa$_1$Xaa$_2$VKCXaa$_3$ISRQCXaa$_4$Xaa$_5$PCKDAGMRFGKCMNGKCHCTPK (SEQ ID NO: 426); wherein

Xaa$_1$ is I or T, Q or E;

Xaa$_2$ is N or D;

Xaa$_3$ is K or R, E, A or Q;

Xaa$_4$ is I, E, L, D, Q, H, V, K or A;

Xaa$_5$ is E K, L, Q, D, V or H; and

the peptide antagonist of Kv1.3 has an optional C-terminal extension of four amino acids.

In some embodiment described herein, the peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NOs: 3, 13, 21, 22, 24, 26, 29, 30, 32, 34, 38, 39, 42-46, 49, 51, 59, 63, 65, 69, 71, 73, 76, 78, 81-83, 85, 87, 89, 92, 96, 101, 103, 104 and 108.

In some embodiment described herein, the antagonist is an isolated fusion protein comprising a peptide antagonist of Kv1.3 conjugated to a half-life extending moiety, wherein the peptide antagonist of Kv1.3 comprises the sequence GVPXaa$_1$Xaa$_2$VKCXaa$_3$ISRQCXaa$_4$Xaa$_5$PCKDAGMRFGKCMNGKCHCTPK (SEQ ID NO: 427); wherein

Xaa$_1$ is I or T;

Xaa$_2$ is N or D;

Xaa$_3$ is K or R;

Xaa$_4$ is I or E; and

Xaa$_5$ is E or K; and

the peptide antagonist of Kv1.3 has an optional C-terminal extension of four amino acids.

In some embodiment described herein, the peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NOs: 3, 22, 34 or 42.

In some embodiment described herein, the C-terminal extension comprises the amino acid sequence of SEQ ID NOs: 123-268.

In some embodiment described herein, the C-terminal extension comprises the amino acid sequence of SEQ ID NOs: 128, 143, 155, 188, 206-210, 212, 214, 216, 219, 223, 224, 227, 230, 232 -235, 237, 239, 240, 243, 252, 261-263, or 268.

The OdK2 variant fusion proteins of the invention are more potent and selective when compared to the fusion protein of native OdK2 sequence, such as KV1C2 (parent KV1C2 fusion protein) of SEQ ID NO: 425. Exemplary fusion proteins of the invention are those comprising OdK2 variant peptides of SEQ ID NOs: 3, 22, 34 or 42 conjugated to human serum albumin (HSA) via a linker $AS(AP)_{20}GS$ (SEQ ID NO: 116).

The parent KV1C2 fusion protein has an IC_{50} of about 13 nM ($1.3x10^{-8}$ M) for inhibiting potassium currents in whole cell patch clamp studies in CHO cells transfected with human Kv1.3, and an IC_{50} value of about 21.4 nM ($2.14x10^{-8}$ M) for inhibiting thallium flux in cells expressing Kv1.3 using FLIPR® Tetra instrument (Molecular Devices). The OdK2 variant fusion protein of the invention as described herein is "equally potent or more potent" Kv1.3 inhibitor when the IC_{50} value in the patch clamp assay described in the materials and methods is about 13 nM ($1.3x10^{-8}$ M) or less, for example $1.0x10^{-8}$ M, $5.0x10^{-9}$ M, $1.0\ x10^{-9}$ M, $5.0\ x10^{-10}$ M, $1.0\ x10^{-10}$ M, $5.0\ x10^{-11}$ M, $1.0\ x10^{-11}$ M, $5.0\ x10^{-12}$ M, $1.0\ x10^{-12}$ M or less, or the IC_{50} value in the thallium flux assay described in the materials and methods is about 21.4 nM ($2.14x10^{-8}$ M) or less, for example $1.0x10^{-8}$ M, $5.0x10^{-9}$ M, $1.0\ x10^{-9}$ M, $5.0\ x10^{-10}$ M, $1.0\ x10^{-10}$ M, $5.0\ x10^{-11}$ M, $1.0\ x10^{-11}$ M, $5.0\ x10^{-12}$ M, $1.0\ x10^{-12}$ M or less. The IC_{50} values for patch clamp and thallium flux for exemplary fusion proteins are shown in Figure 8.

The OdK2 variant and OdK2 variant fusion proteins of the invention as described herein are selective for Kv1.3. Selectivity can be assessed against Kv1.1 using the ratio of an IC_{50} value for Kv1.1 to an IC_{50} value for Kv1.3 for an OdK2 variant fusion protein or OdK2 variant. Selectivity can be further tested against other Kv channels, such as Kv1.2, Kv1.4, Kv1.5, and against hERG, KCa3.1, or Nav1.5 using standard methods. The exemplary OdK2 variant fusion proteins of the invention as described herein can have substantially selectivity for Kv1.3 against Kv1.1, for example 100, 200, 300, 400, 500, 1000, 2000, 3000, 4000, 5000, 6000 or at least 7000 fold selectivity. The parent KV1C2 fusion protein is 68-fold more selective towards human Kv1.3 when compared to human Kv1.1, therefore, the exemplary OdK2 variant fusion proteins of the invention as described herein can have substantially enhanced selectivity, for example about 1.5, 3, 4.5, 6, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or at least 105 fold improved selectivity when compared to the KV1C2 fusion protein. The presence of glutamic acid at position corresponding to Xaa5 in SEQ ID NO: 426 has been observed to improve selectivity.

Residue positions 4, 5, 9, 15 and 16 (residue numbering according to native OdK2 peptide of SEQ ID NO: 1) can be substituted in the native OdK2 to improve both potency and selectivity of the resulting variants and their fusion proteins. The residue positions can be substituted with any amino acid residue as long as the resulting OdK2 variant or its fusion protein, in the above whole cell patch clamp assay or thallium flux assay retains an IC_{50} of about 13 nM ($1.3x10^{-8}$ M) or 21.4 nM ($2.14x10^{-8}$), respectively, or less, and has selectivity (expressed as a ratio of IC_{50} values obtained using patch clamp as described above) for Kv1.3 against Kv1.1 of at least 100. The amino acid sets that can be used for diversification at each selected position include amino acid residues TIQE at position 4, ND at position 5, REAKQ at position 9, ELDIQHVKA at position 15, and KELQDVH at position

16. A glutamic acid (E) at position 15 is associated with increased selectivity for Kv1.3. The substitution G10I is associated with improved selectivity and/or improved affinity for Kv1.3 (residue numbering according to SEQ ID NO: 1). Diversification of OdK2 and its fusion proteins using the amino acid sets described above has resulted in variants displaying improved binding affinity and improved binding selectivity for Kv1.3 when compared to the native peptide or its fusion protein. In another diversification scheme, the amino acid sets that can be used for diversification at each selected position include amino acid residues IT at position 4, ND at position 5, KR at position 9, IE at position 15, and EK at position 16. The resulting variants and/or their fusion proteins can be assessed for selectivity, potency, binding affinity and binding selectivity using well known assays and the ones described within. Exemplary OdK2 variants and their fusion proteins with improved potency and selectivity are variants of SEQ ID NOs: 3, 22, 34 and 42, and their human serum albumin or Fc fusion proteins. Exemplary OdK2 variants with improved binding affinity and %Binding selectivity are variants of SEQ ID NOs: 3, 13, 21, 22, 24, 26, 29, 30, 32, 34, 38, 39, 42-46, 49, 51, 59, 63, 65, 69, 71, 73, 76, 78, 81-83, 85, 87, 89, 92, 96, 101, 103, 104 and 108.

Additional OdK2 variants and OdK2 variant fusion proteins are within the scope of the invention. For example, substitutions can be made in the native OdK2 peptide to positions other than positions 4, 5, 9, 15 and 16 as long as the resulting OdK2 variant and the OdK2 variant fusion protein retains similar selectivity and potency towards Kv1.3 when compared to the parent molecule. Exemplary modifications are for example conservative substitutions that will result in OdK2 variant fusion proteins with similar characteristics to those of the parent molecules. Conservative replacements are those that take place within a family of amino acids that are related in their side chains.

Genetically encoded amino acids can be divided into four families: (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine); (3) nonpolar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); and (4) uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. Alternatively, the amino acid repertoire can be grouped as (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine histidine), (3) aliphatic (glycine, alanine, valine, leucine, isoleucine, serine, threonine), with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic (phenylalanine, tyrosine, tryptophan); (5) amide (asparagine, glutamine); and (6) sulfur-containing (cysteine and methionine) (Stryer (ed.), Biochemistry, 2nd ed, WH Freeman and Co., 1981). Non-conservative substitutions can be made to the native OdK2 peptide that involves substitutions of amino acid residues between different classes of amino acids to improve properties of the OdK2 variants and OdK2 variant fusion proteins. Whether a change in the amino acid sequence of a polypeptide or fragment thereof results in a functional homolog can be readily determined by assessing the ability of the modified polypeptide or fragment to produce a response in a fashion similar to the unmodified polypeptide or fragment using the assays described herein. Peptides, polypeptides or proteins in which more than one replacement has taken place can readily be tested in the same manner. Exemplary additional OdK2 variants and/or OdK2 variant fusion proteins having substitutions resulting in enhanced binding or binding specificity are those having the amino acid sequence of SEQ ID NOs: 4-12, 14-20, 23, 25, 27, 28, 31, 33, 35-37, 40, 41, 47, 48, 50, 52-58, 60-62, 64, 66-68, 70, 72, 74, 75, 77, 79, 80, 84, 86, 88, 90, 91, 93-95, 97-100, 102, 105-107, 109 and 110.

The OdK2 variants (i.e. antagonists according to the invention) as described herein can be fused to a half-life extending moiety to form fusion proteins of the invention. Exemplary half-life extending moieties that can be used include well known human serum albumin, transthyretin (TTR), a thyroxine-binding globulin TGB), albumin-binding domains, or an Fc or fragments thereof. Biologically suitable polymers or copolymers can also be used, for example ethylene glycol, polyethylene glycol (PEG) molecules, such as PEG5000 or PEG20000, dextran, polylysine, fatty acids and fatty acid esters of different chain lengths, for example laurate, myristate, stearate, arachidate, behenate, oleate, arachidonate, octanedioic acid, tetradecanedioic acid, octadecanedioic acid, docosanedioic acid, and the like, polylysine, octane, or carbohydrates (dextran, cellulose, oligo- or polysaccharides.

In another embodiment, the half-life extending moiety of the fusion protein described herein is human serum albumin, albumin binding domain (ADB), or polyethylene glycol (PEG).

In another embodiment, the half-life extending moiety of the fusion protein described herein is human serum albumin.

In another embodiment, the half-life extending moiety of the fusion protein described herein is conjugated to the peptide antagonist of Kv1.3 via a linker.

In another embodiment, the linker of the fusion protein described herein comprises the amino sequence of SEQ ID NOs: 112-122.

The half-life extending moiety can be conjugated directly to the OdK2 variant peptide antagonist of the invention or indirectly via a linker. Exemplary peptide linkers that can be used in fusion proteins of the invention as described herein are linkers having the amino acid sequence of SEQ ID NOs: 112-122. Non-peptide half-life extending moieties can be conjugated directly to the OdK2 variant using well known chemical coupling methods. For example, OdK2 variants can be pegylated using known methods and those described in U.S.

Pat. No. US8043829. Peptide or protein half-life extending moieties can be linked to the peptide during translation of the nucleic acid encoding the fusion protein, as explained in more detail below.

OdK2 variants incorporating half-life extending moieties may be compared for functionality by several well known assays. For example, pharmacokinetic properties of OdK2 variants coupled to PEG or human serum albumin may be evaluated in well known *in vivo* models.

The OdK2 variant fusion proteins of the invention as described herein may be engineered to incorporate a C-terminal extension of four amino acids to the C-terminus of the Odk2 variant before conjugation of the extended peptide to a half-life extending moiety. By not wishing to be bound by any theory, it is believed that extending the C terminus of the OdK2 variant peptide in the fusion proteins would allow for increased binding interactions of the peptide with the extracellular loops of the Kv1.3 channel and increased potency. Exemplary OdK2 fusion proteins with C-terminally extended peptide portion are shown in Figure 6, Figure 7A and Figure 8. The fusion proteins with C-terminally extended peptide portion are typically more potent Kv1.3 inhibitors when compared to the corresponding fusion proteins without the extension. IC_{50} values for exemplary C-terminally extended variants described herein can be about $1x10^{-8}$ M or less, for example about $1x10^{-9}$ M or less, about $1x10^{-10}$ M or less, about $1x10^{-11}$ M or less, or about $1x10^{-12}$ M or less as measured in thallium flux assay described below. Exemplary C-terminal extensions are those shown in SEQ ID NOs: 123-268.

In another embodiment, the isolated fusion protein of the invention comprises:

the peptide antagonist of Kv1.3 of SEQ ID NOs: 3, 22, 34 or 42;

optionally the C-terminal extension of SEQ ID NOs: 128, 143, 155, 188, 206- 210, 212, 214, 216, 219, 223, 224,

227, 230, 232, 235, 237, 239, 240, 243, 252, 261-263, or 268;

the linker of SEQ ID NO: 116 or SEQ ID NO:119; and

human serum albumin as the half-life extending moiety.

In another embodiment, the isolated fusion protein of the invention comprises

the peptide antagonist of Kv1.3 of SEQ ID NO: 42;

the linker of SEQ ID NO: 116; and

human serum albumin as the half-life extending moiety.

In another embodiment, the isolated fusion protein of the invention comprises

the peptide antagonist of Kv1.3 of SEQ ID NO: 42;

the C-terminal extension SEQ ID NO: 209;

the linker of SEQ ID NO: 116; and

human serum albumin as the half-life extending moiety.

In another embodiment, the isolated fusion protein of the invention comprises

the peptide antagonist of Kv1.3 of SEQ ID NO: 3;

the C-terminal extension of SEQ ID NO: 235;

the linker of SEQ ID NO: 116; and

human serum albumin as the half-life extending moiety.

In another embodiment, the isolated fusion protein of the invention comprises

the peptide antagonist of Kv1.3 of SEQ ID NO: 42;

the C-terminal extension of SEQ ID NO: 235;

the linker of SEQ ID NO: 116; and

human serum albumin as the half-life extending moiety.

In another embodiment, the isolated fusion protein of the invention as described herein is at least 100 fold more selective towards human Kv1.3 than towards human Kv1.1, when selectivity is measured as a ratio of an IC_{50} value of the isolated fusion protein for Kv1.1 to an IC_{50} value of the isolated fusion protein for Kv1.3 in a patch clamp assay in cells transfected with Kv1.1 and Kv1.3, respectively.

In another embodiment, the isolated fusion protein of the invention as described herein inhibits potassium currents

with an IC_{50} value at least about 10 fold less than an IC_{50} value for a parent KV1C2 fusion protein of SEQ ID NO: 425 in a patch clamp assay in cells transfected with human Kv1.3.

In another embodiment, the isolated fusion protein of the invention as described herein inhibits potassium currents with an IC_{50} value of about $1.5x10^{-8}$ M or less in a patch clamp assay in cells transfected with human Kv1.3.

In another embodiment, the isolated fusion protein of the invention as described herein inhibits *in vitro* thallium flux with and IC_{50} value of about $2.2x10^{-8}$ M or less in cells transfected with human Kv1.3.

Another embodiment of the invention is an isolated fusion protein comprising a peptide antagonist of Kv1.3 conjugated to a half-life extending moiety via a linker, the peptide antagonist of Kv1.3 having an optional C-terminal extension of four amino acids, wherein

the peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NOs: 3-110;

the C-terminal extension comprises the amino acid sequence of SEQ ID NOs: 123-268;

the linker comprises the amino acid sequence of SEQ ID NOs: 116 or 119; and

the half-life extending moiety is human serum albumin.

Another embodiment of the invention is an isolated peptide antagonist of Kv1.3 comprising the sequence $GVPXaa_1Xaa_2VKCXaa_3ISRQCXaa_4Xaa_5PCKDAGMRFGKCMNGKCHCTPK$ (SEQ ID NO: 426); wherein

Xaa_1 is I or T, Q or E;

Xaa_2 is N or D;

Xaa_3 is K or R, E, A or Q;

Xaa_4 is I, E, L, D, Q, H, V, K or A;

Xaa_5 is E K, L, Q, D, V or H; and

the peptide antagonist of Kv1.3 has an optional C-terminal extension of four amino acids.

In another embodiment the isolated peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NOs: 3, 13, 21, 22, 24, 26, 29, 30, 32, 34, 38, 39, 42-46, 49, 51, 59, 63, 65, 69, 71, 73, 76, 78, 81-83, 85, 87, 89, 92, 96, 101, 103, 104 and 108.

Another embodiment of the invention is an isolated peptide antagonist of Kv1.3 comprising the sequence GVPXaa$_1$Xaa$_2$VKCXaa$_3$ISRQCXaa$_4$Xaa$_5$PCKDAGMRFGKCMNGKCHCTPK (SEQ ID NO: 427); wherein

Xaa$_1$ is I or T;

Xaa$_2$ is N or D;

Xaa$_3$ is K or R;

Xaa$_4$ is I or E; and

Xaa$_5$ is E or K; and

the peptide antagonist of Kv1.3 has an optional C-terminal extension of four amino acids.

In another embodiment the isolated peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NOs: 3, 22, 34 or 42.

Another embodiment of the invention is an isolated peptide antagonist of Kv1.3 comprising the sequence of SEQ ID NOs: 3-110.

The OdK2 variant polypeptides and their fusion proteins of the invention may be produced by chemical synthesis, such as solid phase peptide synthesis, on an automated peptide synthesizer. Alternatively, the polypeptides of the invention can be obtained from polynucleotides encoding the polypeptides by the use of cell-free expression systems such as reticulocyte lysate based expression systems, or by standard recombinant expression systems. Those skilled in the art will recognize other techniques for obtaining the polypeptides of the invention.

Generation of the OdK2 variants is typically achieved at the nucleic acid level. The polynucleotides can be synthesized using chemical gene synthesis according to methods described in U.S. Pat. No. US6521427 and US6670127,

utilizing degenerate oligonucleotides to generate the desired variants, or by standard PCR cloning and mutagenesis. Libraries of variants can be generated by standard cloning techniques to clone the polynucleotides encoding the OdK2 variants into the vector for expression.

The OdK2 variant fusion proteins are typically made by standard molecular biology approaches.

The OdK2 variants and their fusion proteins are tested for their ability to inhibit Kv1.3 using methods described herein. An exemplary assay is an assay measuring inhibition of thallium influx into the cells in cells overexpressing Kv1.3 using FLIPR® Tetra instrument (Molecular Devices). Another exemplary assay employs electrophysiological recordings to measure ionic flux across the cell membrane using well known patch clamp techniques and described herein.

Another embodiment of the invention is an isolated polynucleotide comprising a polynucleotide encoding the OdK2 variant and OdK2 variant fusion protein of the invention.

The polynucleotides of the invention may also comprise at least one non-coding sequence, such as transcribed but not translated sequences, termination signals, ribosome binding sites, mRNA stabilizing sequences, introns and polyadenylation signals. The polynucleotide sequences may also comprise additional sequences encoding additional amino acids. These additional polynucleotide sequences may, for example, encode a marker or well known tag sequences such as a hexa-histidine or a HA tag which facilitate the purification of fused polypeptides. Certain exemplary polynucleotides are disclosed herein, however, other polynucleotides which, given the degeneracy of the genetic code or codon preferences in a given expression system, encode the antagonists of the invention are also within the scope of the invention. Exemplary polynucleotides are polynucleotides comprising a sequence shown in SEQ ID NOs: 429-430.

Another embodiment of the invention is a vector comprising an isolated polynucleotide encoding the OdK2 variants and their fusion proteins of the invention. The vectors of the invention are useful for maintaining polynucleotides, duplicating polynucleotides, or driving expression of a polypeptide encoded by a vector of the invention in biological systems, including reconstituted biological systems. Vectors may be chromosomal-, episomal- and virus-derived such as vectors derived from bacterial plasmids, bacteriophages, transposons, yeast episomes, insertion elements, yeast chromosomal elements, baculoviruses, papova viruses such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses, picornaviruses and retroviruses and vectors derived from combinations thereof, such as cosmids and phagemids.

In one embodiment of the invention the vector is an expression vector. Expression vectors typically comprise nucleic acid sequence elements that can control, regulate, cause or permit expression of a polypeptide encoded by such a vector. Such elements may comprise transcriptional enhancer binding sites, RNA polymerase initiation sites, ribosome binding sites, and other sites that facilitate the expression of encoded polypeptides in a given expression system. Such expression systems may be cell-based, or cell-free systems well known in the art. Nucleic acid sequence elements and parent vector sequences suitable for use in the expression of encoded polypeptides are also well known. An exemplary plasmid-derived expression vector useful for expression of the polypeptides of the invention comprises an *E. coli* origin of replication, an ampicillin resistance (*Amp*) gene, a CMV promoter, a signal sequence, and a SV40 polyadenlyation site.

Another embodiment of the invention is an isolated host cell comprising a vector of the invention. Exemplary host cells include Archaea cells; bacterial cells such as *Streptococci*, *Staphylococci*, *Enterococci*, *E. coli*, *Streptomyces*, cyanobacteria, *B. subtilis* and *S. aureus*;

fungal cells such as *Kluveromyces*, *Saccharomyces*, Basidomycete, *Candida albicans* or *Aspergillus*; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK293, CV-1, Bowes melanoma and myeloma; and plant cells, such as gymnosperm or angiosperm cells. The host cells in the methods of the invention may be provided as individual cells, or populations of cells. Populations of cells may comprise an isolated or cultured population of cells or cells present in a matrix such as a tissue.

Introduction of a polynucleotide, such as a vector, into a host cell can be effected by methods well known to those skilled in the art. These methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, microinjection, cationic lipid-mediated transfection and electroporation.

Another embodiment of the invention is a method of producing the isolated fusion protein of the invention comprising the steps of culturing the host cell under conditions sufficient for the expression of at least one odK2 variant fusion protein, and recovering the fusion protein expressed by the host cell.

Host cells can be cultured under any conditions suitable for maintaining or propagating a given type of host cell and sufficient for expressing a polypeptide. Culture conditions, media, and related methods sufficient for the expression of polypeptides are well known in the art. For example, many mammalian cell types can be aerobically cultured at 37°C using appropriately buffered DMEM media while bacterial, yeast and other cell types may be cultured at 37°C under appropriate atmospheric conditions in LB media.

In the methods of the invention the expression of the OdK2 variant can be confirmed using a variety of well known methods. For example, expression of a polypeptide can be confirmed using detection reagents, such as antibodies using

for example FACS or immunofluorescent techniques, or using SDS-PAGE or HPLC.

Methods of Treatment

Another aspect of the invention is a method of modulating the activity of Kv1.3 in a biological tissue, the method comprising contacting a biological tissue expressing Kv1.3 with a Kv1.3 modulating amount of an OdK2 variant or its fusion protein of the invention, or a pharmaceutically acceptable salt thereof.

OdK2 variants and OdK2 variant fusion proteins of the invention may be utilized in any therapy where it is desired to treat, reduce or alleviate symptoms of Kv1.3-mediated diseases such as inflammatory and autoimmune diseases, diabetes, obesity or cancers.

The methods of the invention may be used to treat an animal patient belonging to any classification. Examples of such animals include mammals such as humans, rodents, dogs, cats zoo animals and farm animals.

The OdK2 variants and/or the OdK2 variant fusion proteins of the invention may be useful for the prophylaxis and treatment of Kv1.3 mediated conditions, such as inflammatory conditions, allergies and allergic conditions, hypersensitivity reactions, autoimmune diseases, severe infections, and organ or tissue transplant rejection. The OdK2 variants and/or the OdK2 variant fusion proteins of the invention are also useful in the preparation of a medicament for such treatment, wherein the medicament is prepared for administration in dosages defined herein.

One embodiment of the invention is method of suppressing T cell activation in a subject having a condition associated with undesired T cell activation, comprising administering to the subject an effective amount of the isolated fusion protein of the invention to suppress T cell activation.

T cell activation can be measured by well known methods, such as measuring reduction of IL-2 production by T cells.

"Suppressing T cell activation" as used herein refers to the ability of the OdK2 variants and OdK2 fusion proteins of the invention to inhibit and reduce T cell activation by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%.

In another embodiment, the condition associated with undesired T cell activation is an inflammatory condition, an immune and proliferative disorder, rheumatoid arthritis (RA), ankylosing spondylitis, psoriatic arthritis, osteoarthritis, osteoporosis, uveitis, inflammatory fibrosis, scleroderma, lung fibrosis, cirrhosis, inflammatory bowel disease, Crohn's disease, ulcerative colitis, asthma, allergic asthma, allergies, Chronic Obstructive Pulmonary Diseases (COPD), multiple sclerosis, psoriasis, contact-mediated dermatitis, systemic lupus erythematosus (SLE) and other forms of lupus, diabetes, type I diabetes, obesity, cancer, lupus, restenosis, systemic sclerosis, scleroderma, glomerulonephritis, Sjogren syndrome, inflammatory bone resorption, transplant rejection, or graft-versus-host disease.

The Kv1.3 channel is expressed on all subsets of T cells and B cells, but effector memory T cells and class-switched memory B cells are particularly dependent on Kv1.3 (Wulff *et al.*, J Immunol 173:776, 2004). Kv1.3 is overexpressed in Gad5/insulin-specific T cells from patients with new onset type 1 diabetes, in myelin-specific T cells from MS patients and in T cells from the synovium of rheumatoid arthritis patients (Beeton *et al.*, Proc Natl Acad Sci USA 103:17414-9, 2006), in breast cancer specimens (Abdul *et al.*, Anticancer Res 23:3347, 2003) and prostate cancer cell lines (Fraser *et al.*, Pflugers Arch 446:559, 2003). Positive outcomes in animal models with Kv1.3 blockers have been described in hypersensitivity models to ovalbumin and tetanus toxoid (Beeton *et al.*, Mol Pharmacol 67:1369, 2005; Koo *et al.*, Clin Immunol 197:99, 1999), models for multiple sclerosis such as rat adoptive-transfer experimental autoimmune encephalomyelitis (AT-EAE) model (Beeton *et al.*, Proc Natl

Acad Sci USA 103:17414-9, 2006), inflammatory bone resorption model(Valverde *et al.*, J Bone Mineral Res 19:155, 2004), models for arthritis (Beeton *et al.*, Proc Natl Acad Sci 103: 17414, 2006; Tarcha *et al.*, J Pharmacol Exper Therap 342: 642, 2012)and obesity, diabetes and metabolic diseases (Xu *et al.*, Hum Mol Genet 12:551, 2003; Xu *et al.*, Proc Natl Acad Sci 101: 3112, 2004).

Exemplary Kv1.3 mediated conditions that may be treated with the OdK2 variants and/or OdK2 variant fusion proteins of the invention are inflammatory conditions, immune and proliferative disorders, including rheumatoid arthritis (RA), ankylosing spondylitis, psoriatic arthritis, osteoarthritis, osteoporosis, uveitis, inflammatory fibrosis (e.g., scleroderma, lung fibrosis, and cirrhosis), inflammatory bowel disorders (e.g., Crohn's disease, ulcerative colitis and inflammatory bowel disease), asthma (including allergic asthma), allergies, COPD, multiple sclerosis, psoriasis, contact-mediated dermatitis, systemic lupus erythematosus (SLE) and other forms of lupus, diabetes, type I diabetes, obesity and cancer, lupus, restenosis, systemic sclerosis, scleroderma, glomerulonephritis, Sjogren syndrome, inflammatory bone resorption, transplant rejection, and graft-versus-host disease.

Administration of the OdK2 variants and/or OdK2 variant fusion proteins of the invention to the animal models of a particular disease can be used to evaluate the use of the OdK2 variants and/or OdK2 variant fusion proteins to ameliorate symptoms and alter the course of diseases. Animal models that can be used are well known, and include models described above and models such as collagen-induced arthritis (CIA) model, diet-induced obesity model, the 2,4,6-trinitrobenesulfonic acid/ethanol (TNBS)-induced colitis model or the oxazalone model, which induce chronic inflammation and ulceration in the colon (Neurath *et al.*, Intern Rev Immunol 19:51-62, 2000), the adoptive transfer model of naïve $CD45RB^{high}$ CD4 T cells to RAG or SCID mice, the

donor naïve T cells attack the recipient gut causing chronic bowel inflammation and symptoms similar to human inflammatory bowel diseases (Read and Powrie, Curr Protoc Immunol Chapter 15 unit 15.13, 2001), ovalbumin challenge model and methacholine sensitization models (Hessel *et al.*, Eur J Pharmacol 293:401-12, 1995).

Pharmaceutical compositions

The "therapeutically effective amount" of the OdK2 variant and/or OdK2 variant fusion protein effective in the treatment of conditions where suppression of Kv1.3 activity is desirable can be determined by standard research techniques. For example, the dosage of the agent that will be effective in the treatment of an inflammatory condition or autoimmune disease such as lupus, multiple sclerosis or psoriasis can be determined by administering the agent to relevant animal models, such as the models described herein.

In addition, *in vitro* assays can optionally be employed to help identify optimal dosage ranges. Selection of a particular effective dose can be determined (*e.g.*, via clinical trials) by those skilled in the art based upon the consideration of several factors. Such factors include the disease to be treated or prevented, the symptoms involved, the patient's body mass, the patient's immune status and other factors known by the skilled artisan. The precise dose to be employed in the formulation will also depend on the route of administration, and the severity of disease, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses can be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The mode of administration for therapeutic use of the OdK2 peptide variants and/or OdK2 variant fusion proteins of the invention may be any suitable route that delivers the variant to the host. Pharmaceutical compositions of these variants are particularly useful for parenteral

administration, e.g., intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous or intranasal.

The OdK2 variants and/or OdK2 variant fusion proteins of the invention may be prepared as pharmaceutical compositions containing an effective amount of the variant as an active ingredient in a pharmaceutically acceptable carrier. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the active compound is administered. Such pharmaceutical vehicles can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. For example, 0.4% saline and 0.3% glycine can be used. These solutions are sterile and generally free of particulate matter. They may be sterilized by conventional, well-known sterilization techniques (*e.g.*, filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, stabilizing, thickening, lubricating and coloring agents, etc. The concentration of the OdK2 variants and/or OdK2 variant fusion proteins of the invention in such pharmaceutical formulation can vary widely, *i.e.*, from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on required dose, fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 ml sterile buffered water, and between about 1 ng to about 100 mg, *e.g.* about 50 ng to about 30 mg or more preferably, about 5 mg to about 25 mg, of the OdK2 variants and/or their fusion proteins of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain about 250 ml of sterile Ringer's solution, and about 1 mg to about 30 mg and preferably 5 mg to about 25 mg of an antagonist of the invention. Actual

methods for preparing parenterally administrable compositions are well known and are described in more detail in, for example, "Remington's Pharmaceutical Science", 15th ed., Mack Publishing Company, Easton, PA.

The OdK2 variants and/or the OdK2 variant fusion proteins of the invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional protein preparations and art-known lyophilization and reconstitution techniques can be employed.

The present invention will now be described with reference to the following specific, non-limiting examples.

Materials and Methods

Kv Channel Expression Constructs and Cell Lines. cDNAs encoding the various Kv channels and chimeric constructs were cloned using routine methods into mammalian expression vectors. cDNAs cloned and expressed were those encoding human Kv1.3 (hKv1.3) (SEQ ID NO: 418), human Kv1.1 (hKv1.1) (SEQ ID NO: 420), human Kv1.2 (hKv1.2) (SEQ ID NO: 419), human Kv1.5 (hKv1.5) (SEQ ID NO: 421), hKv1.3 E3 loop/hKv1.5 chimera (having human Kv1.5 amino acids 1-455 and 496-613, and Kv1.3 E3 loop amino acids 456-495) (Kv1.3 EC3 loop chimera), hKv1.1 E3 loop/hKv1.5 chimera with N terminal His tag (having His tag amino acids 1-9, hKv1.5 amino acids 10-472 and 513-63, and hKv1.1 E3 loop amino acids 473-512)(Kv1.1 EC3 loop chimera), rat Kv1.3 (rKv1.3) (SEQ ID NO: 422), rat Kv1.1 (rKv1.1) (SEQ ID NO: 423), cynomolgus monkey (*Macaca fascicularis)* channel cynoKv1.3 (SEQ ID NO: 424), hKv1.3/hKv1.5 tail chimera (having human Kv1.5 amino acids 1-250 and 497-593, and Kv1.3 amino acid sequences 251-496 (Kv1.3 tail chimera), and hKv1.1/hKv1.5 tail chimera (having human Kv1.5 amino acids 1-250 and 492-588, and Kv1.1 amino acid sequences 251-491 (Kv1.1 tail chimera). For channel expression in HEK cells, Kv genes were cloned into a CMV

promoter driven expression vector encoding the neomycin resistance marker. HEK 293-F (Invitrogen, Carlsbad, CA) cells were stably transfected and cultured in DMEM 10% FBS and 600 µg/ml Geneticin selection media to generate clonal cell lines that expressed Kv channels using standard techniques. For CHO stable expression, CHO-TREx cells (Invitrogen) were stably transfected with pcDNA4/TO-Kv1.x using standard techniques to generate clonal cell lines that expressed each potassium channel in a tetracycline-inducible manner. The culture medium was Ham's F-12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 µg/ml blasticidin and 200 µg/ml zeocin. In some experiments, transient transfection using Lipofectamine 2000 in CHO cells was used. For electrophysiological experiments, cells were co-transfected with an expression vector expressing a truncated CD4 for expression control (pMACs4.1, Milteni Biotech). Assays were performed 24-48 hours after transfection.

Protein Expression and Purification.

The chimera library was expressed as peptide-Fc fusions or a peptide-HSA fusion. The library was initially transfected and expressed in HEK 293-E cells in 48-well or 96-well format. The cells were cultured in DMEM, 10% FBS and 250 µg/ml of Geneticin for selection. For 48-well expression 0.5 ml/well of 3.0 x 10^5 cells/ml were plated in the 48-well plates. The library was transfected using Lipofectamine 2000 using routine methods utilizing 300 ng of plasmid DNA, 25 µl OptiPRO™ SFM media, and 2.4 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The next day, the transfection media was aspirated and 0.5ml of 293 FreeStyle™ media (Invitrogen, Carlsbad, CA) was added to each well. The cells were then incubated for an additional 96 hours before the supernatant was collected and filtered through a 0.2µm filter (Varian).

For 96-well transfection the cells were spun down at 500xg for 5 min, the supernatant was removed and the cells

were re-suspended in 293 FreeStyle™ media and plated into a 96-well plate at 0.6 X 10^6 cells/ml in 0.2ml/well. The library was transfected with the same method as the 48-well transfection.

HEK 293-F cells were used for all small and pilot scale transfections.

Small scale expressions of peptide-Fc fusions were batch purified using Protein A Sepharose 4FF resin using routine methods. Briefly, 20 ml of clarified expression supernatant was mixed with about 0.5 ml of resin equilibrated in DPBS, pH 7.2, and mixed at room temperature for no less than 1 hour. The Protein A resins were washed with 1ml DPBS, pH 7.2, and the bound protein was eluted with 450 µl of 0.1 M sodium acetate, pH 3.0, neutralized with 50 µl of 2M tris, pH 7.0 and dialyzed against 1x DPBS, pH 7.2 overnight at 4°C.

Pilot scale expressions were affinity purified on the AKTA Xpress™ chromatography system (GE Healthcare). Expression supernatants from transiently transfected HEK293-F cells were harvested 4 days after transfection, clarified by centrifugation at 6000 rpm and filtered (0.2 µm PES membrane, Corning, Acton, MA). The relative amount of peptide-Fc fusion was determined with the Octet instrument (ForteBio) using a control toxin-Fc fusion protein spiked into spent medium to generate the standard curve. Samples were then diluted with 10x PBS, pH 7.0 to a final concentration of 1x PBS, pH 7.0 and again filtered (0.2 µm PES membrane). Diluted supernatants were loaded onto a HiTrap MabSelect Sure Protein A column (GE Healthcare) pre-equilibrated with PBS, pH 7.0, at a relative concentration of ~10 mg protein per ml of resin. After loading, the column was washed with PBS, pH7.0 and protein eluted with 10 column volumes of 0.1 M Na-Acetate, pH 3. The protein fractions were neutralized immediately by elution into tubes containing 2.0 M Tris, pH 7 at 20% fraction volume. Peak fractions were pooled and concentrated using centrifugal ultrafiltration devices (Millipore) with 10k MWCO membranes. Concentrated samples

were passed over a Superdex 200 (16/60) column (GE Healthcare) equilibrated and run in PBS, pH7.0 using an AKTA FPLC. Peak fractions were analyzed by non-reducing SDS-PAGE and fractions containing monomeric protein were pooled. Protein concentrations were determined by absorbance at 280nm and 310nm on a BioTek *SynergyHT*™ spectrophotometer. If necessary, the purified proteins were concentrated with a 10K MWCO centrifugal concentrator (Millipore). The quality of the purified proteins was assessed by SDS-PAGE, analytical size exclusion HPLC (Dionex HPLC system), and endotoxin levels measured (LAL assay). Purified proteins were stored at 4°C.

For peptide-HSA fusions, the supernatants were harvested, clarified and filtered through a 0.2 µm filter. Before loading onto a pre-equilibrated 1mL HisTrap column, 10x DPBS was added to a final concentration of 1x. Protein was eluted using a step gradient of imidazole. Fractions containing fusions were collected and analyzed by SDS-PAGE. Fractions containing the protein of interest were pooled and concentrated and run on a Superdex 200 26/60 column. Again, fractions were collected and analyzed by SDS-PAGE. Fractions containing the monomer and dimer of peptide-HSA fusions were pooled separately for the final product. The purified protein was analyzed as described above and stored at 4°C.

Peptide Fusion Protein Direct Binding Assay ("Binding Assay").

Peptide-Fc Fusion proteins. All cell culture reagents were obtained from Invitrogen. Adherent HEK 293F cells stably transfected with plasmids expressing various Kv channels were cultured in DMEM supplemented with 10% FBS and 600 µg/ml Geneticin. Single cell suspensions of Kv channel HEK cells were prepared by rinsing adherent cultures with 1x PBS, then rinsing cultures with 0.25% trypsin EDTA and resuspending cells in cold 1x PBS supplemented with 2% FBS (FACS buffer)

to a final density of $2x10^6$ cells/ml, and dispensing 100 µl/well into 96 well V bottom polypropylene plates (Costar). From this point on the procedure was performed on ice or at 4°C. Cells were centrifuged at 450xg for 2 minutes and supernatants were decanted. 100 µl of peptide-Fc samples in spent Freestyle 293 media or in FACS buffer normalized to 16 nM were added to the cell pellets in designated wells and mixed. To differentiate specific binding and non-specific background, a 10-fold molar excess of synthetic ShK peptide (Bachem) was added to negative control reactions to compete binding of the peptide-Fc fusion protein. Reactions were incubated for 60-90 minutes at 4°C. Cells were washed in 200 µl FACS buffer, and then incubated for 1 hour at 4°C with 100 µl of Goat Fab'2 anti human Fc Cy5 conjugated antibody (Jackson ImmunoResearch Inc.) diluted 1:200 in FACS buffer. Cells were washed in 200 µl FACS buffer, and then resuspended with 100 µl of BD Cytofix™ fixation buffer (BD Biosciences) and stored over night at 4°C. Reactions were read on the FACSArray 96 well auto-sampler flow cytometer (BD Biosciences). Data was analyzed in FlowJo software (Treestar) to obtain geometric mean fluorescence intensities (Geo. MFI) for each reaction. For primary screening binding assays Geo. MFIs for each variant were compared directly to the transiently transfected wild type Odk2-Fc fusion (KV1C2) control and reported as % parent.

Peptide-HSA Fusion Proteins. The assays were performed identically to the direct binding assay for the peptide-Fc fusions except for following: cells were suspended to a final density of $1x10^6$ cells/ml before dispensing 100 µl/well into 96 well V bottom polypropylene plates (Costar), and 50 µl of peptide-HSA fusion samples were added to the cell. The HSA fusions were detected using 50 µl of goat anti-human HSA biotin conjugate (AbCam cat # ab40378) diluted to 2 µg/ml and premixed with streptavidin-PE conjugate () 1:200 in FACS

buffer. Cells were washed in 150 µl FACS buffer, resuspended in 50 µl of BD Cytofix™ fixation buffer (BD Biosciences) and incubated at 4°C for 30 minutes. Reactions were read and data analyzed as described above. For primary screening binding assays Geo. MFIs for each variant were compared directly to the control KV1D261_26 fusion protein (peptide 261 conjugated to HSA via $GS(G_4S)_8$ linker (SEQ ID NO: 120) and reported as %Binding.

Competitive Binding Assay ("Competitive binding"). All cell culture reagents were obtained from Invitrogen. Adherent HEK 293F cells stably transfected with Kv channel expression constructs were cultured in DMEM supplemented with 10% FBS and 600 µg/ml Geneticin. Single cell suspensions of Kv channel HEK cells were prepared by rinsing adherent cultures with 1x PBS, then rinsing cultures with 0.25% trypsin EDTA, then resuspending cells in cold 1x PBS supplemented with 2% FBS (FACS buffer) to a final density of $1x10^6$ cells/ml, and dispensing 100 µl/well into 96 well V bottom polypropylene plates (Costar). From this point on the procedure was performed on ice or at 4°C. Cells were centrifuged at 450xg for 2 minutes and supernatants were decanted. 45 µl of peptides or peptide fusion protein samples in spent Freestyle 293 media or in FACS buffer were added to the cell pellets in designated wells and mixed. Reactions were incubated for 30 minutes at 4°C. 5 µl of 100 nM Agitoxin-2-Cys-TAMRA (Alomone labs) in cell culture media or FACS buffer were added to each well followed by mixing, and reactions were incubated for 60 minutes at 4°C. 200 µl of FACS buffer was added to each well as a wash step, and cells were centrifuged at 450xg for 2 minutes and supernatants were decanted. Cells were resuspended with 50 µl of FACS buffer and reactions were read on the FACSArray 96 well auto-sampler flow cytometer (BD Biosciences). Data was analyzed in FlowJo software (Treestar) to obtain geometric mean fluorescence intensities (Geo. MFI or GMFI) for each reaction. For concentration

response curves, the change in Geo. MFI values across a range of concentrations for each compound were plotted in Graphpad Prism and IC_{50} and Ki values derived using nonlinear regression with a sigmoidal dose-response (variable slope) curve. For calculating Ki, an Agitoxin-2-Cys-TAMRA Kv1.3 KD value of 0.20 nM was assigned (David Triggle (eds). Voltage-Gated Ion Channels as Drug Targets, Volume 29, Page 216, Tab. 7.2.2).

Thallium flux assay. The Kv constructs were stably expressed in HEK293F under G418 selection. Culture medium was HyQ DME/high glucose supplemented with 10% FBS and 600 µg/mL G418. Cells were plated at 10K cells per well into poly-lysine coated 384-well microtiter plates then incubated for 12-36 hours at37°C. Cell plates were washed with assay buffer using a Biotek EL405 (4 cycles, aspirate to 25µL/well, then add 100µL/well). The assay buffer contained (in mM): 130 mM NaCl,4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose. FluxOR dye (Invitrogen) was dissolved per manufacturers' instructions in assay buffer plus 2mM probenecid, and then added to the cells. Cells were stained for 30 minutes at room temperature in the dark. The dye was then washed off with assay buffer. Test compounds were prepared at 2X the test concentration in assay buffer plus 0.2% bovine serum albumin (BSA) and 2mM probenicid. After adding 25µL/well of the test compound solution, the cells were incubated for 30 minutes at room temperature in the dark. Thallium dye fluorescence was monitored in Tetra (Molecular Devices) as the cells were challenged by adding 20 µL/well of stimulus buffer. The stimulus buffer contained 180 mM HEPES, 90 mM KOH, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 1 mM Tl_2SO_4. The fluorescence change was measured 20 seconds after adding the agonist. Data were normalized to the average responses of control wells (N=16 each of 10nM ShK for full inhibition controls, and buffer-only for zero inhibition controls).

T cell Inhibition Assay ("T cell inhibition assay"). Inhibition of IL-2 secretion was used as an indicator for T cell inhibition. Cryopreserved purified primary normal human $CD4^+$ and $CD8^+$ T cells (AllCells LLC) were thawed and suspended in RPMI 1640 (Invitrogen) supplemented with 1% normal human A/B serum (Valley Biomedical Prod & Srv Inc.) at a final density of $2x10^6$ cells/ml, and 100 µl of cells were dispensed into 96 well flat bottom tissue culture plates (NUNC). For peptide-HSA fusion proteins, purified normal human serum albumin (SIGMA) was added to the cell culture media at a final concentration of 3% in order to maintain a constant HSA concentration throughout the concentration-response experiments. Peptide fusion proteins and controls were diluted in cell culture media, and 50 µl/well were added to T cell cultures and incubated for 30 minutes at 37°C/5% CO_2. T cells were activated using anti-human CD3/CD28 T cell expansion beads (Miltenyi Biotec) diluted in cell culture media at a 1:1 bead to cell ratio. Cultures were incubated for ~16 hours at 37°C/5% CO_2, and the supernatants were harvested into 96 well V bottom polypropylene plates (Costar) and clarified by centrifugation. Clarified supernatants were analyzed for IL-2 levels by a chemiluminescent immunoassay derived from the human IL-2 Quantikine Kit (RnD Sytems). Final IL-2 levels were plotted in Graphpad Prism, and IC_{50} values were derived using nonlinear regression with a sigmoidal dose-response (variable slope) curve fit. Some experiments were done at single point 5 nM, 100 nM or 250 nM peptide fusion protein concentration.

Tetanus Toxoid (TTX) T cell Assay. Human PBMC were purified from tetanus toxoid vaccinated healthy donor blood by step gradient centrifugation using Ficoll Pague (GE Healthcare Life Science). PBMC at 10^6 cells/well were stimulated for 3 days in 96 well flat bottom culture plate with 3ug/ml tetanus toxoid (Univ. of Massachusetts Biologic) in RPMI medium

supplemented with 2% human serum, 2 mM glutamine, 1mM sodium pyruvate, 10mM HEPES, 1mM MEM nonessential amino acid solution, and 100 U/ml each of penicillin G and streptomycin (Life Technologies). Culture supernatants were collected on day 2 culture and cell proliferation was measured by overnight pulse with 1uCi/well of ^{3}H-thymidine (Perkin Elmer). Proliferating cells with incorporated radioactive thymidine were harvested onto glass fiber filter plates (Perkin Elmer) and soaked in scintillant (Perkin Elmer) for counting of radioactivity using a Topcount (Packard). Cytokines in supernatants were measured with the MSD detection technology (Meso Scale Discovery).

Electrophysiology. Transfected CHO or HEK cells, $CD4^{+}$ or $CD8^{+}$ T cells were used in electrophysiology. Cells were plated at low density onto glass coverslips. On the day of the experiment, glass cover slips were placed in a bath on the stage of an inverted microscope and perfused (approximately 1 ml/min) with extracellular solution of the following composition: 137 mM NaCl, 2 mM $CaCl_2$, 5.4 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, 0.1% bovine serum albumin, pH 7.4. Pipettes were filled with an intracellular solution of the following composition: 40 mM KCl, 100mM KF, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.3 to 7.4, and had a resistance of 2 to 4 MΩ. All recordings were made at room temperature (22-24°C) using a Multiclamp 700A amplifier and pClamp 9 software (Axon Instruments). Transiently transfected CHO cells were identified using anti-CD4 coated beads (Dynabeads, InVitrogen). Outward potassium currents were measured using the whole-cell configuration of the patch-clamp technique at a test potential of 20 – 40mV from a holding potential of -80mV. The liquid junction potential was calculated to be 7.1mV at 20°C and voltage commands were not corrected. Current records were acquired at 2 - 5 KHz and filtered at 1 - 2 KHz. Currents were elicited once every 20s and were allowed to stabilize for 5 – 10mins prior to recording.

Compounds were applied using an SF-77B Fast-Step Perfusion device (Warner Instruments). 1 – 4 concentrations of compound were tested per cell.

Data analysis:

Concentration- or dose-response data were fitted by non-linear regression (Graph Pad Prism, version 4.0) using the following four parameter general logistic equation:

Response = Basal + (Max – Basal) / [1 + 10 $^{(\log EC_{50} - \text{Log Agonist}) \text{ Hill slope}}$]

Potency was expressed as the - log 10 of the concentration producing 50% maximal effect (pIC_{50} or pEC_{50}).

Peptide Synthesis. Fmoc-Lys(Boc)-Wang resin (0.47 mmol/g substitution) was obtained from Peptide International and pseudoproline dipeptide, Fmoc-Ile-Ser(ΨMeMe pro)-OH was obtained from Novabiochem. All other amino acids were obtained from Applied Biosystems (ABI) or Anaspec. Reagents for automated solid phase peptide synthesis (SPPS) were obtained from ABI. Other reagents required for chemical synthesis were purchased from Sigma/Aldrich. Peptide synthesis was performed on Fmoc-Lys(Boc)-Wang resin (222 mg, 0.104 mmol) via SPPS using an ABI Model 433A automated peptide synthesizer. The standard 0.1-mmole-scale FastMoc MonPrevPeak protocols for HBTU/HOBt/DIEA activation were used according to the manufacturer's protocol. Pseudoproline dipeptide, Fmoc-Ile-Ser(Ψ^{MeMe} pro)-OH, was incorporated at the position shown in bold and underlined in the sequence GVPINVKCKISRQCIEPCKDAGMRFGKCMNGKCHCTPK-resin (SEQ ID NO: 42). The amino-acid side-chain functionality was protected as follows: Arg(Pmc), Asn(Trt), Asp(OtBu), Cys(Trt),Glu(OtBu), Gln(Trt), Lys(Boc), Ser(tBu) and Thr(tBu).

Peptide was cleaved from the resin in (TFA (20 mL), phenol (1.5 g), 1,2 Ethanedithiol (4.0 mL) thioanisole (1.0 mL), water (1.0 mL) and triisopropylsilane (1.0 mL)) for six hours at ambient temperature. The resin was removed via

filtration and rinsed with additional TFA (2 mL). The filtrates were combined and the peptide was precipitated with precooled ethyl ether (400 mL). The peptide isolated by filtration, washed with diethyl ether, and dried in vacuo gave 370.0 mg of crude, linear product: (GVPINVKCKISRQCIEPCKDAGMRFGKCMNGKCHCTPK; SEQ ID NO: 42). The crude linear peptide was oxidized at a peptide concentration 100 µg/mL in 0.1 M Tris-HCL, 1.0 M Guanidine-HCL, 1.0 mM EDTA, 3.0 m ; M glutathione-reduced and 0.3 mM Glutathione-oxidized at ambient temperature. The reaction was stopped after 25 h by drop wise addition of glacial acetic acid to reduce the pH to 3.9 and the peptide was frozen and lyophilized. The crude peptide was purified by Vydac C-18 RP-HPLC. Analytical RP-HPLC, capillary electrophoresis and LC/MC confirmed the purity and molecular mass.

Example 1

Characterization of wild type OdK2 and OsK1 peptides and their fusion proteins

Wild type OdK2 (SEQ ID NO: 1) and OsK1 (SEQ ID NO: 2) peptides were cloned and expressed as IgG4 Fc fusion proteins using the linker $GS(G_4S)_4$ (SEQ ID NO: 119) using routine methods and as described above. The resulting fusion proteins were named KV1C2 (OdK2-Fc fusion) and KV1N2 (OsK1-Fc fusion), respectively. Native OdK2 peptide was isolated and purified from the venom of the Iranian scorpion Odonthobuthus was obtained from Professor Jan Tytgat at the University of Leuven, and recombinant OsK1 peptide from Alomone labs. The native peptides and their fusion proteins were characterized for their binding to Kv1.3, Kv1.3 potency and selectivity using electrophysiology, ability to inhibit T cell activation, and in pharmacokinetic studies.

Binding to Kv1.3 expressing cells

Binding assays were conducted as described above in stable cells expressing the hKv1.3 EC3 loop chimera. KV1C2

(OdK2-Fc fusion) produced a signal in the FACS assay that was 12.8-fold over background and KV1N2 (OsK1-Fc fusion) produced signal 133.0-fold over background (Figure 2), suggesting a high level of binding to the expressed Kv1.3 channel. The binding appeared specific since no binding to human Kv1.3 EC3 loop chimera expressing cells was observed in the presence of a 10-fold excess ShK. The binding was also selective, since KV1C2 (OdK2-Fc fusion) and KV1N2 (OsK1-Fc fusion) did not bind to human Kv1.5 expressing cells.

Electrophysiology

Whole-cell patch clamp studies were performed on CHO cells transfected with human Kv1.3, Kv1.1, Kv1.2 and Kv1.5 ion channels. Osk1 and Odk2 both potently inhibited Kv1.3 currents. OsK1 peptide was significantly more potent than OdK2 against Kv1.3, but the fold selectivity over Kv1.1 was similar for the two peptides. KV1C2 (OdK2-Fc fusion) and KV1N2 (OsK1-Fc fusion) were about 30 - 100-fold less potent towards Kv1.3 when compared to the native peptides. However, KV1C2 selectivity (calculated as Kv1.1 IC_{50} divided by Kv1.3 IC_{50}) was improved about 3 - 4 fold relative to the native peptide. The IC_{50} values and selectivity ratios determined in by electrophysiology are shown in Table 1.

Table 1.

Protein	Electrophysiology		Fold selectivity**	$CD4^+$ T cell inhibition IC_{50} (nM)	$CD8^+$ T cell inhibition IC_{50} (nM)
	Kv1.3 IC_{50} (nM)	Kv1.1 IC_{50} (nM)			
OdK2	0.10	1.90	19	NA	NA
OsK1	0.01	0.21	15	0.03	0.03
OdK2-linker-Fc*	13.00	865.00	67	69.92	69.54
OsK1-linker-Fc*	0.30	2.00	7	2.79	NA

*Linker = $GS(G_4S)_4$, SEQ ID NO: 119

**IC_{50}(Kv1.1)/IC_{50}(Kv1.3)

NA = not done

Inhibition of T-cell activation

KV1C2 (OdK2-Fc fusion) blocked Kv1.3 cellular currents in the Jurkat T cell line, primary $CD4^+$ human T cells (isolated from normal human donors), and Kv1.3 transfected HEK and CHO cells. KV1C2 (OdK2-Fc fusion) also blocked cytokine production from primary human $CD4^+$ and $CD8^+$ T cells activated with anti-CD3/CD28. KV1N2 (OsK1-Fc fusion) blocked Kv1.3 cellular currents in the Jurkat T cell line, competed agitoxin2-cys-TAMRA binding to cells expressing the hKv1.3 EC3 loop chimera, inhibited thallium flux from cells expressing the hKv1.3 EC3 loop chimera, and was tested for its ability to inhibit CD4+ T cell activation. Table 1 shows the obtained IC_{50} values from manual patch-clamp electrophysiology. KV1C2 (OdK2-Fc fusion) also inhibited T cell proliferation upon activation with mitomycin C treated autologous antigen presenting cells displaying tetanus toxoid antigen in an assay described above(Figure 3).

Half life of OdK2-Fc fusion protein

Sparague Dawley Rats were dosed with KV1C2 (OdK2-Fc fusion) through intravenous bolus administration of a 2 mg/ml stock in 1xPBS pH7.0 at 5ml/kg for a final dose of 10 mg/kg. The plasma concentrations were determined by an anti-Fc ELISA

or by FACS as described above. The KV1C2 half-life ($T_{1/2}$) in rats was 60 hours.

Example 2

Generation of OdK2 chimera peptide Fc fusions ("OdK2/Osk1 chimera library" or "KV1C2L1")

The native OdK2 and OsK1 toxin peptide sequences have a high degree of sequence similarity, with divergence at 9 amino acid residues (Figure 1). In order to create peptide variants having enhanced Kv1.3 potency and Kv1.x subtype selectivity, a combinatorial library of peptide-linker-Fc variants using a $GS(G_4S)_4$ linker (SEQ ID NO: 119) was generated in which the OdK2 peptide amino acid sequence was variegated at 8 of 9 positions the OdK2 sequence diverged from OsK1 (positions 3, 4, 5, 9, 10, 12, 16 and 20) in OdK2, SEQ ID NO:1). Position 15 was not included in the library diversification, because of the similarity between isoleucine and leucine at this position. The positions were diversified using OdK2 and OsK1 amino acid residues present at each position. Thus, position 3 was diversified with PI, and positions 4, 5, 9, 10, 12, 16 and 20 with TI, DN, RK, GI, RP, EQ, and KD, respectively. The library design also incorporated six variants with a lysine substitution at position 16, based on previous reports that this mutation increased the potency of the OsK1 peptide (Mouhat *et al.*, Biochem J 385:95-104, 2005), and a glutamine substitution at position 38 as a result of an initial discrepancy in the correct OdK2 amino acid sequence. Thus, the OdK2/Osk1 chimera library consisted of 264 total members including the lysine substitution variants, the OdK2 K38Q variant, and both parent molecules KV1C2 (Odk2 fusion) and (OsK1 fusion). Position numbering is according to the native OdK2 peptide sequence of SEQ ID NO: 1.

The library was generated and expressed using routine molecular biology methods and as described above.

The library was screened using crude supernatants for binding to hKv1.3 using a HEK cell line transfected with the hKv1.3 EC3 loop chimera, and for selectivity by binding to a HEK cell line transfected with the hKv1.1 EC3 loop chimera channel as described above. In the primary screen, binding was measured as % binding of control KV1C2 (%Binding) and selectivity as a ratio of %Binding to Kv1.3 to %Binding to Kv1.1. Figure 4 shows the amino acid sequences, %Binding, selectivity, and IC_{50} values from thallium flux assays for select variants obtained from the OdK2/Osk1 chimera library (KV1C2L1 library) as well as from the amino acid scan library (KV126L1 library) described in Example 4.

Select fusion proteins demonstrating $\geq$ 80% binding to Kv1.3 and $\geq$1.3 fold selectivity over Kv1.1 when compared to the parent KV1C2 (OdK2-Fc fusion) were characterized further.

Example 3

Characterization of OdK2 chimera peptide Fc fusions.

Select OdK2 chimera peptide Fc fusion proteins identified in Example 2 were purified as described above and characterized in secondary binding assays, electrophysiology and T cell inhibition assays.

Electrophysiology

Select variants were assessed for their potency and selectivity in whole cell patch-clamp studies using stably transfected CHO as described above. Inhibition of human Kv1.3 or human Kv1.1 was assessed at a single concentration (1 nM for Kv1.3 or 100nM for Kv1.1) of purified variant (Table 2). Selected variants had significantly increased activity against Kv1.3 but similar activity against Kv1.1 relative to the parent KV1C2.

IC_{50} values were derived from manual patch-clamp electrophysiology studies for select OdK2 chimera peptide Fc fusions using CHO cells stably transfected with either human Kv1.3 or Kv1.1 as described in materials and methods. Fold

selectivity was calculated as a ratio of IC_{50}(Kv1.1) to IC_{50}(Kv1.3). Table 3 shows IC_{50} values for select variants and the parent OdK2 and OsK1 fusions KV1C2 and KV1N2, respectively.

Table 2.

Fusion protein	Kv1.3 %Inhibition @ 1nM	Kv1.1 %Inhibition @100nM
KV1D197	83	37
KV1D37	67	40
KV1D267	64	49
KV1D229	85	54
KV1D261	85	61
KV1D161	76	63
KV1D69	86	64
KV1C2*	36	40
KV1D280	-	97 **

* parent (OdK2-Fc fusion)

**fusion protein at 10 nM

Table 3.

Fusion protein	Kv1.3 IC_{50} (nM)*	Kv1.1 IC_{50} (nM)*	Selectivity**
KV1D261	0.15	85	582
KV1D197	0.3	145	483
KV1D229	0.25	70	280
KV1D267	0.47	103	219
KV1C2	13	865	67
KV1N2	0.3	2	7

*IC_{50} values from patch clamp epectrophysiology

**selectivity = $IC_{50(Kv1.1)}/IC_{50(Kv1.3)}$

Based on the patch-clamp studies, the OdK2 chimera peptide Fc fusion proteins had about from 28 to about 87 fold improved Kv1.3 potency and about 3 to 9-fold improved selectivity when compared to the parent KV1C2 (OdK2-Fc fusion), and about ~85-fold improved selectivity when compared to the parent KV1N2 (OsK1-Fc fusion).

Inhibition of T cell activation

Select OdK2 chimera peptide Fc fusions were characterized for their ability to inhibit T cell activation measured as inhibition of IL-2 secretion from anti-CD3/C28-induced T cells as described in materials and methods. Inhibition was measured at a single concentration (100nM) of OdK2 chimera peptide Fc fusions and results are presented as % inhibition of maximum IL-2 production in Figure 5A. KV1B03 is identical to the parent KV1C2 (OdK2 fusion) and was incidentally recreated during library construction. Figure 5B shows concentration-dependent inhibition of IL-2 production by activated T-cells by KV1D261. The IC_{50} value for $CD4^+$ T cell inhibition for the KV1D261 fusion protein was 1.66 nM.

Correlation of inhibition of T cell activation and the binding to Kv1.3 using crude supernatants conducted during primary screen of the library was evaluated to assess ability to identify functional Kv1.3 blockers with the binding assay (Figure 5C). Significant correlation was identified between the two assays (Pearson's correlation r 0.9339, $p<0.0001$).

Antagonistic effects of fusion proteins are attributable to the peptide portion

To determine whether the inhibitory characteristics as well as the selectivity of the OdK2 chimera peptide Fc fusions were retained by the peptide portion, select OdK2

chimera fusions were compared in their characteristics with the corresponding synthetic peptides.

KV1D261 and the corresponding synthetic peptide p261 (SEQ ID NO: 42) were characterized for Kv1.3 potency and selectivity, cross-reactivity to rat Kv1.3 channels, and inhibition of T cell activation. hERG was assayed using routine methods such as those referenced in Dubin *et al.*, J Biomol Screen. 10:168-81, 2005. All cell lines used stably expressed the channel of interest except for rat Kv1.3 and hKCa3.1. Results of the experiments for KV1D261 and the corresponding synthetic peptide p261 are shown in Table 4. The synthetic peptide was ~10-fold more potent than the Fc fusion by both electrophysiology and T cell inhibition, and retained selectivity when compared to the corresponding fusion protein, demonstrating that the engineered peptide region is responsible for conveying the properties of potency and selectivity. The loss of potency when fused with the Fc was expected due to increased entropy with increased molecular weight.

Table 4.

Channel/cell line	Assay	KV1D261		synthetic p261	
		IC_{50} (nM)	Fold selectivity*	IC_{50} (nM)	Fold selectivity*
human CD4+ T-cell	IL-2 Inhibition	~2	-	0.02	-
human hKv1.3/CHO	ephys	0.15	-	0.02	-
rat Kv1.3/CHO	ephys	<0.3	-	-	
Kv1.3/human CD4+ T-cell	ephys	<1	-	0.016	-
Kv1.3/rat CD4+ T-cell	ephys	0.29	-	-	
hKv1.1/CHO	ephys	85	567x	3.3	165
hKv1.2/CHO	ephys	>300	>2000	68	3400
hKv1.5/CHO	ephys	>1000	>6000	-	-
hERG/CHO	ephys	>1000	>6000	-	-
KCa3.1/human CD4+ T-cell	ephys	>100	>600	-	-
KCa3.1/CHO	ephys	-	-	>100	>5000

ephys: electrophysiology manual patch-clamp

*Ratio $IC_{50(hKv1.x)}/IC_{50(hKv1.3)}$ in CHO cells

Pharmacokinetic properties of KV1D261

Sprague Dawley Rats were dosed with KV1D261 (261-Fc fusion) through intravenous bolus administration of a 8.3 mg/ml stock in 1xPBS pH7.0 at 1.2ml/kg for a final dose of 10 mg/kg. The protein was measured using both ELISA and FACS assay. The half life was assessed to be about 72 hours.

Example 4

Generation of an amino acid scanning library (KV1D26L1 library)

To further improve selectivity of Kv1.3 blocking peptides, a scanning library was designed by single substitutions of 9 amino acids (A, R, Q, E, H, L, K, V, D) at each non-cysteine residue of the peptide region of KV1D26 (corresponding peptide p26, SEQ ID NO: 111), a potent but non-selective variant identified from the OdK2/Osk1 chimera library described in Example 2. This amino acid scanning library consisted of 270 variants.

The library was generated and expressed using routine molecular biology methods and as described above. Briefly, the genes encoding for the variant peptides were synthesized using synthetic gene assembly (U.S. Pat. No. US6521427 and U.S. Pat. No. US6670127) and cloned in frame with the GS $(G_4S)_4$ (SEQ ID NO: 119) linker IgG4 Fc fusion partner in a mammalian expression vector.

The library was expressed as above and screened as crude supernatants for binding to an HEK cell line transfected with the human Kv1.3 EC3 loop chimera channel, and for selectivity by binding to an HEK cell line transfected with the human Kv1.1 EC3 loop chimera channel as described above. Activity was normalized following quantitation of each variant. %Binding for Kv1.3 and Kv1.1 was expressed as a percentage of parent KV1C2 (OdK2-Fc fusion) as described in materials and methods. The library was also screened in the thallium flux

assay using the two cell lines as described in materials and methods.

Figure 4A shows the sequences of select variants. Binding and thallium flux data are summarized in Figure 4B.

Multiple variants from the amino acid scanning library demonstrated increased binding and selectivity for Kv1.3 over Kv1.1. From the binding assay screen, lysine to glutamine substitutions consistently resulted in increased selectivity for Kv1.3 over Kv1.1. Select variants demonstrating $\geq$ 80% binding to Kv1.3 and $\geq$1.3 fold selectivity over Kv1.1 were purified and characterized further.

Example 5

Characterization of variants obtained from the amino acid scanning library (KV1D26L1 library)

Competition with Kv toxin inhibitors

Select variants were purified and assessed for their potential to compete with the known Kv1.3 inhibitor, agitoxin-2-CysTAMRA, in single point and concentration-response assays as described above. Inhibition was assessed in stable HEK293 cells expressing the human Kv1.3 EC3 loop chimera using 10 nM Agitoxin-2-CysTAMRA and each variant at either 40 nM for single point readings or from 0.015 nM to 4 µM for the concentration-response studies.

% inhibition of agitoxin-2-CysTAMRA binding and IC_{50} values for select variants are shown in Table 5. Select variants inhibited binding of agitoxin-2-CysTAMRA to Kv1.3 at levels similar to KV1D261, and IC_{50} values ranged from 5 nM to 1.3 µM, also with several of the variants in the low nanomolar range of KV1D261.

Table 5.

Protein ID	Competitive binding % inhibition Single Concentration (40nM)	Competitive Binding IC_{50} (nM)	% T cell Inhibition Single Concentration (5 nM)	% T cell Inhibition Single Concentration (250 nM)	T cell Inhibition IC_{50} (nM)
KV1D664	65.1	5.7	0	71.1	NA
KV1D625	21.3	NA	0	70.1	NA
KV1D581	61.2	NA	13.4	78	NA
KV1D603	64.2	NA	33.7	77.5	7.5
KV1D342	47.6	NA	7.5	78.2	NA
KV1D576	76.2	NA	16.3	76.2	NA
KV1D291	49.6	NA	0	NA	NA
KV1D579	31.1	94.4	0	NA	26.8
KV1D294	46.8	NA	0	NA	NA
KV1D662	45.4	NA	0	NA	NA
KV1D665	5.6	1394	0	NA	NA
KV1D656	30.4	91.1	0	NA	NA
KV1D414	32.2	NA	0	NA	NA
KV1D356	69.7	NA	22.2	NA	NA
KV1D437	69.8	5	16.8	NA	NA
KV1D604	26.5	NA	22.4	NA	26.9
KV1D261	69.7	7.5	67.9	NA	2.3
No inhibition	0	0	NA	NA	0
Fc Control	NA	NA	0	NA	0

NA: not done

0: no measurable inhibition

Inhibition of T cell activation

The ability of select variants to inhibit T cell activation was assessed as described above using IL-2 secretion as a marker for activation.

Assays were performed at single variant concentration of either 5 nM or 250 nM or using a range from 0.015 nM to 250 nM for a concentration response. %Inhibition from maximal signal for select variants are shown in Table 5.

KV1D579 is a potent and selective Kv1.3 inhibitor

KV1D579 was studied in whole cell patch clamp studies and thallium flux using HEK cells transfected with human

Kv1.3, Kv1.1, or Kv1.6 as described above. IC_{50} values for KV1D579 are listed in Table 6 together with the parent KV1D26, an OdK chimera-Fc fusion variant isolated in Example 2. Values in parenthesis are derived from thallium flux assays and values not in parenthesis are derived from the patch clamp study.

Table 6.

Protein	hKv1.3 IC_{50} (nM)	hKv1.1 IC_{50} (nM)	Selectivity *	hKv1.6	
				IC50 (nM)	Selectivity*
KV1D26	~ 0.21/(0.32)	~2/(5.8)	~10/(18)	0.6	3
KV1D579	0.14/(0.14)	>1000/(>380)	>7143/(>2714)	93.7	669

Values in parenthesis are derived from thallium flux inhibition assays

* ratio of $IC_{50(Kv1.x)}/IC_{50(Kv1.3)}$

Example 6

Generation of C-terminal extension library (KV1D819L1 library)

Several peptide-Fc fusion proteins conjugated using the linker $GS(G_4S)_4$ (SEQ ID NO: 119) were found to induce undesired inflammatory cytokine release from cultures of resting human peripheral blood mononuclear cells *in vitro*, while the corresponding synthetic peptides themselves did not. Therefore the undesired cytokine release was attributable to the format of the bivalent peptide-Fc fusion or the Fc itself.

To prevent unwanted cytokine release, peptide 261 (p261) (SEQ ID NO: 42) was synthesized as a human serum albumin fusion protein using linker $GS(G_4S)_4$ (SEQ ID NO: 119).

The potency of the resulting fusion protein (KV1D261_23) was further optimized by generating a fusion protein library based on KV1D261_23, where the peptide p261 was extended at its C-terminus by four amino acids. It was hypothesized that

extending the C terminus of the peptide region of KV1D261_23 fusion protein would allow for increased binding interactions of the peptide with the extracellular loops of the Kv1.3 channel and thereby increasing potency.

The KV1D261_23 fusion protein was modified by inserting 4 additional amino acids between the C terminus of the peptide and the intervening $GS(G_4S)_4$ linker (SEQ ID NO: 119), with the following 12 residues per position in full combination (Q, R, P, H, K, T, N, S, E, G, A, and D) at new positions 39, 40, 41 and 42 in the 261 peptide. The ratio for each residue was 1, except R was 1.5, and S was 0.5, and the resulting theoretical number of possible variants of this library was 12^4 (20,736). Genes coding for the peptides with variant C terminal extensions were synthesized as previously described and cloned in frame with the $GS(G_4S)_4$ linker (SEQ ID NO: 119) and human serum albumin fusion partner in a mammalian expression vector using routine molecular biology methods.

The library was expressed recombinantly in transiently transfected HEK293 cells. Crude supernatants were screened using direct binding assays and functional thallium flux assays using an HEK cell line transfected with the human Kv1.3 tail chimera channel for Kv1.3 potency, and an HEK cell line transfected with the human Kv1.1 tail chimera channel. Hits from this library were high in basic (R, H, and K), acidic (T, N and G), and non-polar (A and P) residues. Figure 6 shows the results of the binding and thallium flux assays for select fusion proteins.

Example 7

Characterization of C-terminal extension library (KV1D819L1 library)

Select candidates identified from the C-terminal extension library were purified and binding to Kv1.3 confirmed. Concentration-response curves were generated for the thallium flux assays. Table 7 shows the IC_{50} values

determined in the thallium flux assay and the amino acid sequence of the C-terminal extension for each variant. KV1D261_26 (p261-HSA fusion using $GS(G_4S)_8$ linker (SEQ ID NO: 120)) was used as a control in the assay. Most p261 C-terminal extension peptide fusions demonstrated similar or increased potency in the thallium flux when compared to the control with a non-extended peptide moieties. KV1D261_26 fusion having longer linker than KV1D261_23 consistently tested ~5 fold more potent than KV1D261_23 (p261-HSA fusion protein using $Gs(G_4S)_4$ linker (SEQ ID NO: 119).

Table 7.

Protein ID	Half-life extending moiety	Linker	Peptide portion	C-terminal extension		%Binidng (of KV1D261_26)	Thallium Flux IC_{50} (nM)
				Amino acid sequence	SEQ ID NO:		
KV1D826	HSA*	$GS(G_4S)_4$**	p261 extended	TRRP	156	109.8%	3.4
KV1D829	HSA	$GS(G_4S)_4$	p261 extended	AHRH	209	96.6%	3.3
KV1D830	HSA	$GS(G_4S)_4$	p261 extended	AQRP	210	76.2%	7.6
KV1D831	HSA	$GS(G_4S)_4$	p261 extended	ARRN	234	117.2%	2.7
KV1D832	HSA	$GS(G_4S)_4$	p261 extended	ASDN	236	15.1%	37.2
KV1D834	HSA	$GS(G_4S)_4$	p261 extended	ATRP	206	95.2%	5.9
KV1D841	HSA	$GS(G_4S)_4$	p261 extended	NHRT	222	88.4%	5.9
KV1D848	HSA	$GS(G_4S)_4$	p261 extended	PNRT	223	66.2%	6.7
KV1D853	HSA	$GS(G_4S)_4$	p261 extended	PTTR	241	55.0%	9.1
KV1D856	HSA	$GS(G_4S)_4$	p261 extended	RHNT	226	34.5%	6.7
KV1D858	HSA	$GS(G_4S)_4$	p261 extended	RKKP	173	73.7%	6.0
KV1D860	HSA	$GS(G_4S)_4$	p261 extended	RQTR	253	52.6%	6.7
KV1D863	HSA	$GS(G_4S)_4$	p261 extended	RRRP	208	100.0%	2.8
KV1D864	HSA	$GS(G_4S)_4$	p261 extended	RTRQ	248	57.9%	5.3
KV1D865	HSA	$GS(G_4S)_4$	p261 extended	SHRP	139	127.6%	2.8
KV1D869	HSA	$GS(G_4S)_4$	p261 extended	TTRT	233	77.4%	5.9
KV1D261_26	HSA	$GS(G_4S)_4$	p261 extended	none		99.8%	1.0

*human serum albumin

**SEQ ID NO: 119

Example 8

Peptide HSA fusion protein engineering

Peptide 261 was engineered as fusion protein with human serum albumin (HSA) using various linkers and the resulting

fusion proteins tested for their Kv1.3 potency and selectivity, and inhibition of T cell activation. Results of T cell inhibition measured as inhibition of IL-2 secretion as well as thallium flux inhibition with peptide 261 conjugated to HSA via various linkers is shown in Table 8.

Table 8.

Protein	Linker name	Linker SEQ ID NO:	Tcell Inhibition IC_{50} (nM)	Thallium Flux KV1.3 IC_{50} (nM)
KV1D261_23	$GS(G_4S)_4$	119	80.1	20.0
KV1D261_ 32	$(EAAAK)_4$	117	129.1	10.2
KV1D261_ 33	$AS(AP)_{10}GS$	115	10.8	1.1
KV1D261_ 34	$AS(AP)_{20}GS$	116	4.5	0.4
KV1D261_ 35	$1DC1(13AA)_2$	113	20.3	3.6
KV1D261_ 36	$1DC1(13AA)_3$	114	10.4	1.8
KV1D261_ 37	1FU1	112	151.1	9.2
KV1D261_ 38	$(EAAAK)_8$	118	10.3	0.7

The linker engineering indicated that inserting the more structured alanine proline (AP) repeat linker into the fusion protein instead of the more flexible glycine serine (GS) linker significantly improved potency, and that increasing the linker length further enhanced potency. The $IDC1(13AA)_2$ (SEQ ID NO: 113) and $IDC1(13AA)_3$ (SEQ ID NO: 114) and $(EAAAK)_8$ (SEQ ID NO: 118) linkers also improved potency, but appeared less stable during protein production with fusion protein fragments present following purification. The fusion protein KV1D261_34 with peptide 261 conjugated to HSA via the $AS(AP)_{20}GS$ linker (SEQ ID NO: 116) had an IC_{50} for Kv1.3 of about 4 nM in T cell inhibition assay and IC_{50} of about 0.4 nM in the thallium flux assay.

Characterization of p261 and p579 HSA fusions

Fusion protein KV1D261_34 and the corresponding synthetic peptide p261 as well as peptide p579 conjugated to human HSA via the $AS(AP)_{20}GS$ linker (SEQ ID NO: 116) to generate a fusion protein KV1G49.KV1W720 were further tested in various functional assays and for their selectivity against human Kv channels as shown in Table 9.

Table 9.

Channel/ cell line		KV1D261_34		p261 synthetic peptide		KV1G49.KV1W720	
	Assay	IC_{50} (nM)	Fold selectivity#	IC_{50} (nM)	Fold selectivity#	IC_{50} (nM)	Fold selectivity
hKv1.3/HEK	competitive binding	54	-	1.53	-	342.0	
hKv1.3/CHO	thallium flux	0.3	-	0.03-0.04	-	9.0	
hKv1.3/CHO	ephys	1.2	-	0.02*	-	3.0	
primary human $CD4^+$ T cell	ephys	NA	-	0.016	-		
primary human $CD4^+$ T cell	T cell inhibition	2.1	-	0.02	-	15.8	
hKv1.1/CHO	thallium flux	79	247	2.0-8.0	5-266	>251**	
hKv1.1/CHO	ephys	>1000	>1000	3.3	165		
hKv1.2/CHO	ephys	>100	>100	68	3400		
hKv1.4/CHO	ephys	>100	>100	>300	>15000		
hKv1.5/CHO	ephys	>100	>100	-	-		
hKv1.6/CHO	ephys	10	8	0.32	12		
hKv1.7/CHO	ephys	>100	>100	>100	>5000		
hKCa3.1/CHO	ephys	-	-	>100	>5000		

ephys: patch clamp electrohpysiology

*IC50 20 pM in 1% BSA and 13 pM in 5% FCS

**incomplete concentration response due to low potency. IC_{50} is reported as greater than the highest concentration tested

#ratio of $IC_{50(KV1.1)}/IC_{50(Kv1.3)}$ in CHO cells

KV1D261_34 was also tested for its ability to inhibit thapsigargin-induced IL-17A production from human and porcine (Yucatan minipig) whole blood. The IC_{50} value for the

inhibition in both humans and minipig was 0.5 nM. KV1D261_34 also inhibited minipig $CD4^+$ T cell activation (IL-2 secretion) with an IC_{50} value of 1.6 nM.

Example 9

Generation and characterization of additional C-terminal extension peptide fusions

Peptides p261 (SEQ ID NO: 42) and p579 (SEQ ID NO: 3) were extended using several C-terminal extensions and conjugated to HSA via the $AS(AP)_{20}GS$ (SEQ ID NO: 116) or the $GS(AP)_{20}AS$ linker (SEQ ID NO: 428). Select fusion proteins were expressed, purified and characterized in assays including competitive binding, thallium flux, inhibition of *in vitro* human $CD4^+$ T cell activation, and electrophysiology assays.

Figure 7A shows characteristics of fusion proteins having various C-terminal extensions on peptide p261. For assessing T cell inhibition, select variants were tested either for % inhibition of anti CD3/CD28 stimulated human $CD4^+$ T cell IL-2 production at a single concentration of 1 nM (T cell %Inhibition @1nM), or IC_{50} values were derived from concentration-response curves using the same assay (T cell Inhibition IC_{50} nM). Fold selectivity was measured as a ratio of IC_{50}(Kv1.1)/IC_{50}(Kv1.3) using the values from the thallium flux assay.

The competitive binding Ki values for the C-terminally extended peptide fusion proteins ranged from about 0.15 nM to about 18.0 nM, compared to the Ki of about 1 nM for the parent KV1D261_34 non-extended peptide fusion. Several of the variants had improved potency and selectivity compared to the parent KV1D261_34, with the thallium flux IC_{50} values ranging from about 10 pM to about 1 nM and fold selectivity over Kv1.1 from about 30 to about 800.

Manual patch-clamp studies were conducted for select peptide fusion proteins in Kv1.3 transfected CHO cell lines as described above. The IC_{50} values for KV1G15.KV1W686 (C-

terminal extension of peptide p261 using AHRH (SEQ ID NO: 209) fused to HSA via $AS(AP)_{20}GS$ linker (SEQ ID NO: 116) was 199 pM. Some of the C-terminal insertions resulted in a ~5-10-fold increase in potency over the parent KV1D261_34.

Fusion proteins of p579 with and without select C-terminal extension conjugated to HSA with an intervening $GS(AP)_{20}AS$ (SEQ ID NO: 116) were characterized in competitive binding (competition with 10 nM agitoxin-cys-TAMRA binding to HEK cells)and T-cell inhibition (IL-2 secretion) and the results are shown in Figure 7B.Some of the C- terminal insertions resulted in a ~3-5-fold increase in potency over the parent, KV1G49.KV1W720.

Several HSA fusion proteins conjugated to peptide variants using the $AS(AP)_{20}GS$ linker (SEQ ID NO: 116) were tested for their ability to induce the secretion of cytokines and chemokines (IFNγ, IL-1β, TNF-α, IL-2, IL-4, IL-5, IL12p70 and IL-13) from PBMCs. No induction was seen for these HSA fusions.

Example 10

KV1D261 34 pharmacokinetics and pharmacodynamics in minipigs

KV1D261_34, (261 peptide HSA fusion protein with intervening $AS(AP)_{20}GS$ linker (SEQ ID NO: 116)) was administered to mini-pigs as a single intravenous injection (30nmoles/Kg). Heparinized plasma samples were collected at various time points post administration and plasma levels were determined using anti-261 capture / anti-penta His-HRP ELISA. Figure 9 shows results as the mean ±SD of 4 animals through day 36, and of 2 animals at the final 54 day time point. Half-life (T1/2) of the fusion protein was 5-7 days, with clearance (CL) 0.008-0.01 ml/min/Kg and volume of distribution (Vss) = 90-120ml/Kg.

Target engagement was assessed by measuring IL-17A secretion from lymphocytes in whole blood ex-vivo. Whole blood samples were collected from each study animal at times -48, -24, -1 hour pre administration, and at time points

between 0.017-1296 hours (54 days) post administration of KV1D261_34 (30nmoles/Kg). Whole blood samples were treated with thapsigargin in the absence or presence of 1 μM exogenous 261 peptide, with each condition in triplicate per sample. IL-17 cytokine levels were measured in an anti-porcine IL-17A ELISA, and %Inhibition by exogenous peptide 261 calculated as follows:

100-(Average IL-17 pg/ml concentrations in the +thapsigargin+ 1μM exogenous 261 reactions/Average IL-17 pg/ml concentrations in the +thapsigargin reactions)x100.

The results of experiment, expressed as the % Inhibition of thapsigargin-induced IL-17 secretion by exogenous 261 peptide (mean ± SD of 4 animals except at 54 days, where 2 animals were analyzed) are shown in Figure 10. The average %Inhibition of thapsigargin-induced IL-17 secretion by exogenous 261 for all of the predosed samples and the non-dosed control animal (all time points) was 75.3±14.1%. The mean %Inhibition of thapsigargin-induced IL-17 secretion by exogenous 261 in whole blood ex-vivo was $\leq$ 25% for approximately 14 days following IV administration of KV1D261-34, indicating a high level of target engagement by circulating KV1D261_34. Plasma concentrations at these time points were >10nM. At later time points the %Inhibition of thapsigargin-induced IL-17 secretion by exogenous 261 increased to >65% on day 36 and reached baseline levels of $\geq$80% by day 54, indicating gradual reduction of target engagement as plasma levels declined. The findings of this study show that KV1D261_34 is stable in plasma *in vivo* and has a long plasma half-life in minipigs. Circulating KV1D261_34 is bioavailable and inhibits thapsigargin-induced IL-17 secretion on lymphocytes following intravenous dosing. Inhibition of thapsigargin-induced IL-17 secretion appeared to be well correlated with plasma concentration and effective plasma concentrations were consistent with the proposed mechanism of action (Kv1.3 block) of KV1D261_34.

Example 11

Minipig keyhole limpet hemocyanin (KLH)-induced delayed type hypersensitivity (DTH) model

Minipig Delayed Type Hypersensitivity (DTH) model was used as an *in vivo* model to assess ability of KV1D261_34 to inhibit T cell function. Minipigs were dosed IV with either vehicle (PBS, n = 6) or KV1D261_34 (30nmoles/kg, n = 6). As a positive control, Cyclosporine A was administered subcutaneously twice daily with 1 ml/kg at a concentration of 10 mg/ml from day -1 until necropsy., (n = 6). Dosing commenced one day prior (Day -1) to immunization with KLH antigen. Minipigs were immunized with KLH on day 0 by 1 ml subcutaneous injections of either5 mg/ml KLH in Incomplete Freund Adjuvant (IFA), or PBS in IFA for the control group. Injections were made at ~5 locations on the caudal aspect of the hind legs. Animals were then challenged on day 7 with intradermal injections of 0.1 ml/spot with KLH at 10, 5, 2.5, 1.25 mg/ml, or PBS, with one spot per challenge dose on the left flank, and a duplicate challenge spot on the right flank. The level of induration was measured on days 9 and 10, one and two days post challenge, and on day 10 tissue and blood samples were collected for additional measurements of draining lymph node cellularity, anti antigen antibody titers, and challenge site histology.

Draining lymph nodes were collected on day 10, two days after the challenge, for the determination of lymph node cellularity. The results are shown in Figure 11. KV1D261_34 treated animals had significantly reduced cellularity when compared to the vehicle treated challenged animals. Cellularity was reduced to a level that was comparable to that observed in the unimmunized control animals.

No significant reduction in anti-KLH antibody titers or induration was detected in KV1D261_34 administered animals at days 9 and 10 (1 and 2 days post-challenge).

Claims

1) An isolated fusion protein comprising a peptide antagonist of Kv1.3 conjugated to a half-life extending moiety, wherein the peptide antagonist of Kv1.3 comprises the amino acid sequence $GVPXaa_1Xaa_2VKCXaa_3ISRQCXaa_4Xaa_5PCKDAGMRFGKCMNGKCHCTPK$ (SEQ ID NO: 427); wherein

a) Xaa_1 is I or T;

b) Xaa_2 is N or D;

c) Xaa_3 is K or R;

d) Xaa_4 is I or E; and

e) Xaa_5 is E or K, and has an IC_{50} value of about $1x10^{-8}$ M or less towards Kv1.3 in a whole cell patch clamp assay on Chinese hamster ovary (CHO) cells transfected with Kv1.3, and is at least l00-fold more selective towards Kv1.3 when compared to a fusion protein comprising a wild—type Odk2 peptide of SEQ ID NO: 1 conjugated to the half—life extending moiety , when selectivity is calculated as a ratio of Kv1.1 IC_{50} value to Kv1.3 IC_{50} value in the whole cell patch clamp assay on CHO cells transfected with Kv1.3 or Kv1.1.

2) The fusion protein of claim 1, wherein the peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NOs: 3, 22, 34 or 42.

3) The fusion protein of claim 2, wherein the half—life extending moiety is human serum albumin (HSA), albumin binding domain (ADB), or polyethylene glycol (PEG).

4) The fusion protein of claim 3, wherein the half—life extending moiety is human serum albumin.

5) The fusion protein of claim 3, wherein the half—life extending moiety is conjugated to the peptide antagonist of Kv1.3 via a linker.

6) The fusion protein of claim 5, wherein the linker comprises the amino sequence of SEQ ID NOs: 112—122 or 428.

7) The fusion protein of claim 6, wherein
a) the peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NOs: 3, 22, 34 or 42;
b) the linker comprises the amino acid sequence of SEQ ID NO: 116 or SEQ ID NO:119; and
c) the half—life extending moiety is human serum albumin.

8) The fusion protein of claim 6, wherein
a)the peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NO: 42;
b)the linker comprises the amino acid sequence of SEQ ID NO: 116; and
c)the half-life extending moiety is human serum albumin.

9) The fusion protein of claim 6, wherein
a)the peptide antagonist of Kv1.3 comprises the amino acid sequence of SEQ ID NO: 42;
b)the C-terminal extension comprises the amino acid sequence of SEQ ID NO: 209;
c)the linker comprises the amino acid sequence of SEQ ID NO: 116; and
d)the half-life extending moiety is human serum albumin.

10) A pharmaceutical composition comprising the fusion protein of claim 2 and a pharmaceutically acceptable carrier.

11) An isolated peptide antagonist of Kv1.3 comprising the amino acid sequence GVPXaa$_1$Xaa$_2$VKCXaa$_3$ISRQCXaa$_4$Xaa$_5$PCKDAGMRFGKCMNGKCHCTPK (SEQ ID NO: 427);

wherein

a)Xaa$_1$ is I or T;

b)Xaa$_2$ is N or D;

c)Xaa$_3$ is K or R;

d)Xaa$_4$ is I or E; and

e)Xaa$_5$ is E or K, wherein the peptide antagonist of Kv1.3 has an IC$_{50}$ value of about 1x10^{-8} M or less towards Kv1.3 in a whole cell patch clamp assay on Chinese hamster ovary (CHO) cells transfected with Kv1.3, and is at least 100-fold more selective towards Kv1.3 when compared to a fusion protein comprising a wild-type Odk2 peptide of SEQ ID NO: 1 conjugated to the half-life extending moiety , when selectivity is calculated as a ratio of Kv1.1 IC$_{50}$ value to Kv1.3 IC$_{50}$ value in the whole cell patch clamp assay on CHO cells transfected with Kv1.3 or Kv1.1.

12) The peptide antagonist of Kv1.3 of claim 11, comprising the amino acid sequence of SEQ ID NOs: 3 or 42.