Abstract:
The present invention relates to the ability of specialized non-naturally occurring peptides to bind to sodium channels and inhibit activation of the sodium channels. The invention further relates to methods for regulating of sodium absorption and fluid volume and treating disorders responsive to modulating sodium absorption by modulating the binding of specialized non-naturally occurring peptides to sodium channels.

**FIG. 1**

A GGL^{24}PVPLDQT^{31}LPLNVNP^{38}A

**SUBSEQUENCE A**

**SUBSEQUENCE B**

**Title:** IMPROVED PEPTIDE INHIBITORS OF SODIUM CHANNELS
Improved Peptide Inhibitors of Sodium Channels

STATEMENT OF PRIORITY

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 62/061,461, filed October 8, 2014, the entire contents of which are incorporated by reference herein.

FIELD OF THE INVENTION

[0002] The present invention relates to optimized peptides that are specialized non-naturally occurring peptides with improved ability to bind to sodium channels and inhibit activation of the sodium channels. The invention further relates to methods for regulating sodium absorption and fluid volume and treating disorders responsive to modulating sodium absorption by activity of sodium channels.

BACKGROUND OF THE INVENTION

[0003] Epithelial mucosal surfaces are lined with fluids whose volume and composition are precisely controlled. In the airways, a thin film of airway surface liquid helps protect mammalian airways from infection by acting as a lubricant for efficient mucus clearance (Hobbs et al, J. Physiol. 591: 4377 (2013), Knowles et al, J. Clin. Invest. 109:571 (2002)). This layer moves cephalad during mucus clearance and excess liquid that accumulates as two airways converge is eliminated by Na⁺-modulated airway surface liquid absorption with Na⁺ passing through the epithelial Na⁺ channel (ENaC) (Hobbs et al, J. Physiol. 591: 4377 (2013), Knowles et al, J. Clin. Invest. 109:571 (2002)). Critically, the mechanism by which ENaC activity is regulated in the airways is poorly understood. Recently, evidence has been accumulating that molecular regulators in the airway surface liquid can serve as volume sensing signals whose dilution or concentration can alter specific cell surface receptors that control ion transport rates to either absorb or secrete airway surface liquid as needed (Chambers et al, Respir. Physiol. Neurobiol. 159:256 (2007)). As one of the regulated targets, ENaC must be cleaved by intracellular furin-type proteases and/or extracellular channel activating proteases (CAPs) such as prostasin to be active and to conduct Na⁺ (Planes et al, Curr. Top. Dev. Biol. 78:23 (2007); Rossier, Proc. Am. Thorac. Soc. 1:4 (2004); Vallet et al, Nature 389:607 (1997); Chraibi et al, J. Gen. Physiol. 111:127 (1998)). ENaC can also
be cleaved and activated by exogenous serine proteases such as trypsin, an action that is attenuated by the protease inhibitor aprotinin (Vallet et al, Nature 389:607 (1997)). When human bronchial epithelial cultures are mounted in Ussing chambers where native airway surface liquid is washed away, ENaC is predominantly active, suggesting that cell attached proteases are predominant (Bridges et al, Am. J. Physiol. Lung Cell. Mol. Physiol. 281:L16 (2001); Donaldson et al, J. Biol. Chem. 277:8338 (2002)). In contrast, under thin film conditions, where native airway surface liquid is present, ENaC activity is reduced, suggesting that airway surface liquid contains soluble protease inhibitors (Myerburg et al, J. Biol. Chem. 281:27942 (2006); Tarran et al, J. Gen. Physiol 127:591 (2006); Gaillard et al., 2010 Pfleugers Arch, 460: 1-17).

[0004] Recently, it has been shown that the Short Palate Lung and Nasal epithelial Clone (SPLUNC1) protein comprises up to 10% of the total protein in the airway surface liquid and can readily be detected in both nasal lavage and tracheal secretions (Bingle, C. D., and Craven, C. J. (2002) PLUNC: a novel family of candidate host defense proteins expressed in the upper airways and nasopharynx Hum Mol Genet 11, 937 ; Campos, M. A., et al. (2004) Purification and characterization of PLUNC from human tracheobronchial secretions Am J Respir Cell Mol Biol 30, 184; Lindahl, M., Stahlbom, B., and Tagesson, C. (2001); Identification of a new potential airway irritation marker, palate lung nasal epithelial clone protein, in human nasal lavage fluid with two-dimensional electrophoresis and matrix-assisted laser desorption/ionization-time of flight Electrophoresis 22, 1795). SPLUNC1 appears to be a volume sensing molecule since it can be secreted onto the mucosal surface of the superficial epithelia where ENaC is expressed (Bartlett et al, J. Leukoc. Biol. 83:1201 (2008); Bingle et al, J. Pathol 205:491 (2005)). Furthermore, SPLUNC1 has been demonstrated to contain a subdomain that functions as an inhibitor of ENaC through its N-terminal domain.

[0005] The present invention discloses novel specialized non-naturally occurring peptides that mimic the properties of SPLUNC1 in regulation of sodium channels by binding to and inhibiting ion transport to regulate sodium absorption and fluid volume and treat disorders responsive to modulating sodium absorption.

SUMMARY OF THE INVENTION

[0006] The present invention is based, in part, on the design of specialized non-naturally occurring peptides to regulate the activity of sodium channels. Accordingly, in
one aspect the invention relates to a method of inhibiting the activation of a sodium channel, comprising contacting a sodium channel with a specialized non-naturally occurring peptide or a functional fragment thereof. In one embodiment, the sodium channel is an epithelial sodium channel (ENaC). In one embodiment, the specialized non-naturally occurring peptide or a functional fragment thereof binds to the sodium channel.

[0007] Another aspect of the invention relates to a method of inhibiting sodium absorption through a sodium channel, comprising contacting the sodium channel with a specialized non-naturally occurring peptide or a functional fragment thereof. In one embodiment, the specialized non-naturally occurring peptide or a functional fragment thereof binds to the sodium channel.

[0008] A further aspect of the invention relates to a method of increasing the volume of fluid lining an epithelial mucosal surface, comprising contacting a sodium channel present on the epithelial mucosal surface with a specialized non-naturally occurring peptide or a functional fragment thereof. In one embodiment, the specialized non-naturally occurring peptide or a functional fragment thereof binds to the sodium channel.

[0009] Another aspect of the invention relates to a method of reducing the level of a sodium channel present on the surface of a cell, comprising contacting the sodium channel with a specialized non-naturally occurring peptide or a functional fragment thereof. In one embodiment, the specialized non-naturally occurring peptide or a functional fragment thereof binds to the sodium channel.

[0010] A further aspect of the invention relates to a method of treating a disorder responsive to inhibition of sodium absorption across an epithelial mucosal surface in a subject in need thereof, comprising delivering to the subject a therapeutically effective amount of a specialized non-naturally occurring peptide or a functional fragment thereof. In one embodiment, the specialized non-naturally occurring peptide or a functional fragment thereof binds to the sodium channel.

[0011] Another aspect of the invention relates to a method of regulating salt balance, blood volume, and/or blood pressure in a subject in need thereof, comprising delivering to the subject a therapeutically effective amount of a specialized non-naturally occurring peptide or a functional fragment thereof. In one embodiment, the specialized non-naturally occurring peptide or a functional fragment thereof binds to the sodium channel.
Another aspect of the invention relates to a specialized non-naturally occurring peptide or a functional fragment thereof that mimics the sodium channel binding domain of a PLUNC protein and binds to a sodium channel, wherein cleavage of the sodium channel by a protease is inhibited when bound to the peptide.

Another aspect of the invention relates to a kit comprising the peptide of the invention.

Another aspect of the invention relates to the use of a specialized non-naturally occurring peptide or a functional fragment thereof for the preparation of a medicament to treat a disorder responsive to inhibition of sodium absorption in a subject in need thereof.

These and other aspects of the invention are set forth in more detail in the description of the invention below.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows the sequence of S18 (SEQ ID NO:1). Highlighted in red are residues essential for ENaC interaction, while highlighted in blue are residues that were found not to contribute.

Figs. 2A-2B show the 1st half (LPVPLDQT (SEQ ID NO:113) but not the remainder (DQTLPLNVNP (SEQ ID NO:114) of S18 is required for inhibition of ENaC and preservation of ASL height.

Fig. 3A shows that S18 (SEQ ID NO:1) and aaLPVPLDQTLPVLNVNPaa (SEQ ID NO:2) have equal potency and efficacy, despite SEQ ID NO:2 being flanked by D-alanines. Fig 3B shows that S18, and aaLPVPLDQTaa (SEQ ID NO:3) have equal potency and efficacy, despite (SEQ ID NO:3) being flanked by D-alanines. Fig. 3A also shows the relative potency of a sample of peptides, including aaLPNlePLDQTaa (SEQ ID NO:5), which displays increased potency, relative to S18 or SEQ ID NO:4.

Fig. 4 shows an additional comparison of S18 and SEQ ID NO:5, and increased potency of SEQ ID NO:5.

Figs. 5A-5U show the results of experiments analyzing the effects of various peptides on internalization of alpha-ENaC in HEK293T cells and effects on cell viability when GFP-tagged alpha-ENaC is co-expressed with beta and gamma ENaC. EC50 values are shown at the bottom of several of the figures. In these figures, ADG is a negative control peptide with the sequence: NH3-ADGGLLLLNNPPPPQTVV-NH2.
The peptides used in these experiments were S18 (SEQ ID NO:1), SEQ ID NO:2, SEQ ID NO:141, SEQ ID NO:129, SEQ ID NO:130, SEQ ID NO:128, SEQ ID NO:131, SEQ ID NO:132, SEQ ID NO:133, SEQ ID NO:134, SEQ ID NO:135, SEQ ID NO:136, SEQ ID NO:137, SEQ ID NO:138, SEQ ID IDNO:131, SEQ ID NO:139; SEQ ID NO:140, SEQ ID NO:144 and SEQ ID NO:127. Also used in these experiments were peptides of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:136, each with an aa cap at both the N- and the C-terminus. Also shown is amiloride, which inhibits ENaC by another mechanism and does not reduce the amount of functional receptor on the surface of cells.

[0021] Fig. 6 shows the results of an experiment analyzing the effects of various peptides on internalization of alpha-ENaC in HEK293T cells when GFP-tagged alpha-ENaC is co-expressed only with gamma ENaC and no beta-ENaC. In this figure, SEQ ID NO:143 (negative control peptide) and water (vehicle) controls are used along with S18 (SEQ ID NO:1) and SEQ ID NO:128. While SEQ ID NO:1 AND SEQ ID NO:128 are effective in reducing alpha-ENaC when beta-ENaC is co-expressed, no effect is observed in this experiment when beta-ENaC is not present.

[0022] Fig. 7A shows the results of an experiment analyzing the effect on percent survival of pENaC-Tg C57BL:FVB mice after treatment with the S18 peptide (SEQ ID NO:1). S18 (100 mM solution) was administered 3 times per day at 1 μL/g of body weight.

[0023] Fig. 7B shows the results of an experiment analyzing the effect on percent survival of pENaC-Tg C57BL:FVB mice after treatment with inhaled SEQ ID NO:142 or SEQ ID NO:128.

[0024] Figs. 7C and 7D show the results of an experiment analyzing the effect on percent survival of pENaC-Tg C57BL:C3H mice after treatment with SEQ ID NO:129 or SEQ ID NO:128.

[0025] Fig. 7E show the results of an experiment analyzing the effect on percent survival (left panel) and weight gain (right panel) of pENaC-Tg C57BL:C3H mice after treatment with a once daily dose of SEQ ID NO:128.

[0026] Fig. 7F shows the results of an experiment analyzing the effect on percent survival of pENaC-Tg C57BL:C3H mice after treatment with SEQ ID NO:127, SEQ ID NO:134, or SEQ ID NO:136.
DETAILED DESCRIPTION OF THE INVENTION

[0027] The present invention will now be described in more detail with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0028] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. All publications, patent applications, patents, patent publications and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

[0029] Amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by either the one-letter code, or the three letter code, both in accordance with 37 C.F.R. §1.822 and established usage.

[0030] As used in the description of the invention and the appended claims, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0031] Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

[0032] The term "consists essentially of" (and grammatical variants), as applied to a peptide sequence of this invention, means a peptide that consists of both the recited sequence (e.g., SEQ ID NO) and a total of ten or less (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) additional amino acids on the N-terminal and/or C-terminal ends of the recited sequence such that the function of the peptide is not materially altered. The total of ten or less additional amino acids includes the total number of additional amino acids on both ends added together. The term "materially altered," as applied to peptides of the invention, refers to an increase or decrease in binding activity (e.g., to a sodium channel or specialized non-naturally occurring peptide) of at least about 50% or more as compared to the activity of a peptide consisting of the recited sequence.
[0033] The term "modulate," "modulates," or "modulation" refers to enhancement (e.g., an increase) or inhibition (e.g., a decrease) in the specified level or activity.

[0034] The term "enhance" or "increase" refers to an increase in the specified parameter of at least about 1.25-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 8-fold, 10-fold, twelve-fold, or even fifteen-fold.

[0035] The term "inhibit" or "reduce" or grammatical variations thereof as used herein refers to a decrease or diminishment in the specified level or activity of at least about 15%, 25%, 35%, 40%, 50%, 60%, 75%, 80%, 90%, 95% or more. In particular embodiments, the inhibition or reduction results in little or essentially no detectible activity (at most, an insignificant amount, e.g., less than about 10% or even 5%).

[0036] The term "contact" or grammatical variations thereof as used with respect to a specialized non-naturally occurring peptide and a sodium channel, refers to bringing the specialized non-naturally occurring peptide and the sodium channel in sufficiently close proximity to each other for one to exert a biological effect on the other. In some embodiments, the term contact means binding of the specialized non-naturally occurring peptide to the sodium channel.

[0037] A "therapeutically effective" amount as used herein is an amount that provides some improvement or benefit to the subject. Alternatively stated, a "therapeutically effective" amount is an amount that will provide some alleviation, mitigation, or decrease in at least one clinical symptom in the subject. Those skilled in the art will appreciate that the therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

[0038] By the terms "treat," "treating," or "treatment of," it is intended that the severity of the subject's condition is reduced or at least partially improved or modified and that some alleviation, mitigation or decrease in at least one clinical symptom is achieved.

[0039] The term "fragment," as applied to a peptide, will be understood to mean an amino acid sequence of reduced length relative to a reference peptide or amino acid sequence and comprising, consisting essentially of, and/or consisting of an amino acid sequence of contiguous amino acids identical to the reference peptide or amino acid sequence. Such a peptide fragment according to the invention may be, where appropriate, included in a larger polypeptide of which it is a constituent. In some embodiments, such fragments can comprise, consist essentially of, and/or consist of
peptides having a length of at least about 4, 5, 6, 7, 8, 9, 10, or more consecutive amino acids of a peptide or amino acid sequence according to the invention. In other embodiments, such fragments can comprise, consist essentially of, and/or consist of peptides having a length of less than about 10, 9, 8, 7, 6, 5, 4, or less consecutive amino acids of a peptide or amino acid sequence according to the invention.

[0040] As used herein, the terms "protein" and "polypeptide" are used interchangeably and encompass both peptides and proteins, unless indicated otherwise.

[0041] A "fusion protein" is a polypeptide produced when two heterologous nucleotide sequences or fragments thereof coding for two (or more) different polypeptides not found fused together in nature are fused together in the correct translational reading frame. Illustrative fusion polypeptides include fusions of a peptide of the invention (or a fragment thereof) to all or a portion of glutathione-S-transferase, maltose-binding protein, or a reporter protein (e.g., Green Fluorescent Protein, β-glucuronidase, β-galactosidase, luciferase, etc.), hemagglutinin, c-myc, FLAG epitope, etc.

[0042] As used herein, a "functional" peptide or "functional fragment" is one that substantially retains at least one biological activity normally associated with that peptide (e.g., binding to or inhibiting a sodium channel). In particular embodiments, the "functional" peptide or "functional fragment" substantially retains all of the activities possessed by the unmodified peptide. By "substantially retains" biological activity, it is meant that the peptide retains at least about 20%, 30%, 40%, 50%, 60%, 75%, 85%, 90%, 95%, 97%, 98%, 99%, or more, of the biological activity of the native polypeptide (and can even have a higher level of activity than the native peptide). A "non-functional" peptide is one that exhibits little or essentially no detectable biological activity normally associated with the peptide (e.g., at most, only an insignificant amount, e.g., less than about 10% or even 5%). Biological activities such as protein binding and sodium channel inhibitory activity can be measured using assays that are well known in the art and as described herein.

[0043] The term "about," as used herein when referring to a measurable value such as an amount of polypeptide, dose, time, temperature, enzymatic activity or other biological activity and the like, is meant to encompass variations of ± 20%, ± 10%, ± 5%, ± 1%, ± 0.5%, or even ± 0.1% of the specified amount.
[0044] A first aspect of the invention relates to the ability of specialized non-naturally occurring peptides to bind to a sodium channel and prevent activation of the sodium channel, thereby inhibiting the flow of sodium ions. Thus, one aspect of the present invention relates to a method of inhibiting the activation of a sodium channel, comprising contacting (e.g., binding) a sodium channel with a specialized non-naturally occurring peptide or a functional fragment thereof. In one embodiment, the sodium channel is an epithelial sodium channel (ENaC), e.g., human ENaC, or a non-human mammalian ENaC. In another embodiment, the sodium channel is one that is similar in sequence and/or structure to ENaC, such as acid-sensing ion channels (ASIC). The inhibition of sodium channel activation can be measured by any method known in the art or disclosed herein, including, without limitation, measuring sodium flow or change in potential across a membrane, across a cell, or across a natural or artificial lining. The inhibition can be at least about 20%, e.g., at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%.

[0045] The method of inhibiting the activation of a sodium channel can be carried out, e.g., on an isolated sodium channel, a sodium channel in an artificial membrane, or a sodium channel in a cell. In one embodiment, the sodium channel is present in an isolated cell, e.g., a cultured primary cell or cell line. In another embodiment, the isolated cell is part of an epithelial cell culture, e.g., a natural or artificial epithelial lining, e.g., a cell culture in a device (such as an Ussing chamber) in which characteristics such as ion flow and/or potential can be measured across lining. In another embodiment, the cell is part of an isolated tissue or a tissue culture. In a further embodiment, the cell can be present in an animal, e.g., an animal that is a disease model or a subject in need of treatment.

[0046] In one embodiment, the step of contacting (e.g., binding) the sodium channel with a specialized non-naturally occurring peptide comprises delivering the specialized non-naturally occurring peptide or a functional fragment or homolog thereof to a cell comprising the sodium channel.

[0047] As used herein, the term "homolog" is used to refer to a polypeptide which differs from a the disclosed specialized non-naturally occurring peptide by modifications to the specialized non-naturally occurring peptide, but which significantly retains a biological activity of the disclosed non-naturally occurring peptide. Minor modifications include, without limitation, changes in one or a few amino acid side chains, changes to one or a few amino acids (including deletions, insertions, and
substitutions), changes in stereochemistry of one or a few atoms, and minor
derivatizations, including, without limitation, methylation, glycosylation,
phosphorylation, acetylation, myristoylation, prenylation, palmitoylation, amidation, and
addition of glycosylphosphatidyl inositol. The term "substantially retains," as used
herein, refers to a fragment, homolog, or other variant of a peptide that retains at least
about 20% of the activity of the naturally occurring peptide (e.g., binding to a sodium
channel), e.g., about 30%, 40%, 50% or more. Other biological activities, depending on
the peptide, may include enzyme activity, receptor binding, ligand binding, induction of
a growth factor, a cell signal transduction event, etc.

[0048] In one embodiment, the method comprises delivering to a cell
comprising a sodium channel an isolated specialized non-naturally occurring peptide. In
exemplary embodiments, the specialized non-naturally occurring peptide comprises,
consists essentially of, or consists of the disclosed specialized non-naturally occurring
peptide or a functional fragment thereof. In another embodiment, the isolated
specialized non-naturally occurring peptide comprises, consists essentially of, or consists
of an amino acid sequence that is at least 70% identical, e.g., at least 75%, 80%, 85%,
90%, 95%, 96%, 97%, 98%, or 99% identical to the publicly known amino acid
sequence or a functional fragment thereof. In some embodiments, the peptides comprise
a portion of the natural amino acid sequence of a PLUNC protein with one or more
conservative substitutions with natural or non-natural amino acids and/or one or more
additions of non-natural amino acids. Conservative substitutions are described below.
In some embodiments, the peptides comprise one or more terminal modifications as
described below.

[0049] Non-limiting examples of peptides of the invention are disclosed in Table
1 below. In some embodiments, the peptides of the invention may comprise one or more
additional residues at the amino- and/or carboxyl-terminal ends. In some embodiments,
the one or more additional residues are D-alanines. For example, a peptide may
comprise one or two D-alanines at the amino- and/or carboxyl-terminal ends.
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Where a = D-alanine, Nle = Norleucine, HYP = 4-hydroxyproline, DHP = 3,4-dehydro-L-proline, Ahp = aminoheptanoic acid, 2PP = (2R,5S)-5-phenylpyrrolidine-2-carboxylic acid, MS = L-a-methylserine, and mV = N-methylvaline
The specialized non-naturally occurring peptides of the invention also include functional portions or fragments. The length of the fragment is not critical as long as it substantially retains the biological activity of the peptide (e.g., sodium channel binding activity). Illustrative fragments comprise at least about 4, 5, 6, 7, 8, 9, 10, or more contiguous amino acids of a specialized non-naturally occurring peptide. In other embodiments, the fragment comprises no more than about 10, 9, 8, 7, 6, 5, or 4 contiguous amino acids of a specialized non-naturally occurring peptide.

Likewise, those skilled in the art will appreciate that the present invention also encompasses fusion polypeptides comprising a specialized non-naturally occurring peptide or a functional fragment thereof. As another alternative, the fusion protein can comprise a reporter molecule. In other embodiments, the fusion protein can comprise a polypeptide that provides a function or activity that is the same as or different from the activity of the peptide, e.g., a targeting, binding, or enzymatic activity or function.

Likewise, it will be understood that the peptides specifically disclosed herein will typically tolerate substitutions in the amino acid sequence and substantially retain biological activity. To identify peptides of the invention other than those specifically disclosed herein, amino acid substitutions may be based on any characteristic known in the art, including the relative similarity or differences of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like.

In identifying amino acid sequences encoding peptides other than those specifically disclosed herein, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (see, Kyte and Doolittle, J Mol. Biol. 757:105 (1982); incorporated herein by reference in its entirety). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, id.), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8);
tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

[0055] Accordingly, the hydropathic index of the amino acid (or amino acid sequence) may be considered when modifying the peptides specifically disclosed herein.

[0056] It is also understood in the art that the substitution of amino acids can be made on the basis of hydrophilicity. U.S. Patent No. 4,554,101 (incorporated herein by reference in its entirety) states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

[0057] As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (±3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

[0058] Thus, the hydrophilicity of the amino acid (or amino acid sequence) may be considered when identifying additional peptides beyond those specifically disclosed herein.

[0059] Peptides (and fragments thereof) of the invention include peptides that have at least about 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or higher amino acid sequence identity with the peptide sequences disclosed herein.

[0060] As is known in the art, a number of different programs can be used to identify whether a polypeptide has sequence identity or similarity to a known sequence. Sequence identity or similarity may be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, *J Mol. Biol.* 48A 43 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 55:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux *et al.*, *Nucl. Acid Res.* 72:387 (1984), preferably using the default settings, or by inspection.

[0061] An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise
alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J Mol. Evol.* 55:351 (1987); the method is similar to that described by Higgins & Sharp, *CABIOS* 5:151 (1989).

[0062] Another example of a useful algorithm is the BLAST algorithm, described in Altschul *et al.*, *Mol. Biol.* 215:403 (1990) and Karlin *et al.*, *Proc. Natl. Acad. Sci. USA* 90:5873 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul *et al.*, *Meth. Enzymol.* 266:460 (1996); blast.wustl.edu/blast/README.html. WU-BLAST-2 uses several search parameters, which are preferably set to the default values. The parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.


[0064] A percentage amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

[0065] The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the peptides specifically disclosed herein, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity of sequences shorter than a sequence specifically disclosed herein, will be determined using the number of amino acids in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as insertions, deletions, substitutions, *etc.*

[0066] In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0," which obviates the need for a weighted scale or parameters as described below for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the
number of matching identical residues by the total number of residues of the "shorter" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

[0067] Peptides and fragments of the invention can be modified for in vivo use by the addition, at the amino- and/or carboxyl-terminal ends, of a blocking agent to facilitate survival of the relevant polypeptide in vivo. This can be useful in those situations in which the peptide termini tend to be degraded by proteases prior to cellular uptake. Such blocking agents can include, without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxyl terminal residues of the peptide to be administered. This can be done either chemically during the synthesis of the peptide or by recombinant DNA technology by any suitable methods. For example, one or more non-naturally occurring amino acids, such as D-alanine, can be added to the termini. Alternatively, blocking agents such as pyroglutamic acid or other molecules known in the art can be attached to the amino and/or carboxyl terminal residues, or the amino group at the amino terminus or carboxyl group at the carboxyl terminus can be replaced with a different moiety. Additionally, the peptide terminus can be modified, e.g., by acetylation of the N-terminus and/or amidation of the C-terminus. Likewise, the peptides can be covalently or noncovalently coupled to pharmaceutically acceptable "carrier" proteins prior to administration.

[0068] In one embodiment, the peptides or fragments thereof of the invention are administered directly to a subject. Generally, the compounds of the invention will be suspended in a pharmaceutically-acceptable carrier (e.g., physiological saline) and administered orally or by intravenous infusion, or administered subcutaneously, intramuscularly, intrathecally, intraperitoneally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, or intrapulmonarily. In another embodiment, the intratracheal or intrapulmonary delivery can be accomplished using a standard nebulizer, jet nebulizer, wire mesh nebulizer, dry powder inhaler, or metered dose inhaler. They can be delivered directly to the site of the disease or disorder, such as lungs, kidney, or intestines. The dosage required depends on the choice of the route of administration; the nature of the formulation; the nature of the patient's illness; the subject's size, weight, surface area, age, and sex; other drugs being administered; and the judgment of the attending physician. Suitable dosages are in the range of 0.01-100.0 µg/kg. Wide variations in the needed dosage are to be expected in view of the variety of peptides, fragments, and homologs available and the differing efficiencies of various routes of
administration. For example, oral administration would be expected to require higher dosages than administration by i.v. injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art. Administrations can be single or multiple (e.g., 2-, 3-, 4-, 6-, 8-, 10-, 20-, 50-, 100-, 150-, or more fold). Encapsulation of the peptides, fragments, and homologs in a suitable delivery vehicle (e.g., polymeric microparticles or implantable devices) may increase the efficiency of delivery, particularly for oral delivery.

[0069] According to certain embodiments, the peptides, or fragments thereof can be targeted to specific cells or tissues in vivo. Targeting delivery vehicles, including liposomes and targeted systems are known in the art. For example, a liposome can be directed to a particular target cell or tissue by using a targeting agent, such as an antibody, soluble receptor or ligand, incorporated with the liposome, to target a particular cell or tissue to which the targeting molecule can bind. Targeting liposomes are described, for example, in Ho et al, Biochemistry 25:5500 (1986); Ho et al, J. Biol. Chem. 262:13979 (1987); Ho et al, J. Biol. Chem. 262:13973 (1987); and U.S. Pat. No. 4,957,735 to Huang et al., each of which is incorporated herein by reference in its entirety).

[0070] Another aspect of the invention relates to a method of inhibiting sodium absorption through a sodium channel, comprising contacting (e.g., binding) the sodium channel with a specialized non-naturally occurring peptide or fragment thereof. Inhibition of sodium absorption can be measured by any technique known in the art or disclosed herein.

[0071] Another aspect of the invention relates to a method of increasing the volume of fluid lining an epithelial mucosal surface, comprising contacting (e.g., binding) a sodium channel present on the epithelial mucosal surface with a specialized non-naturally occurring peptide or a functional fragment or homolog thereof. The volume of fluid lining an epithelial mucosal surface can be measured by any technique known in the art or disclosed herein.

[0072] A further aspect of the invention relates to a method of reducing the level of a sodium channel present on the surface of a cell, comprising contacting (e.g., binding) the sodium channel with a specialized non-naturally occurring peptide or a functional fragment or homolog thereof.

[0073] An additional aspect of the invention relates to a method of treating a disorder responsive to inhibition of sodium absorption across an epithelial mucosal
surface in a subject in need thereof, comprising delivering to the subject a therapeutically
effective amount of a specialized non-naturally occurring peptide or a functional
fragment or homolog thereof. In one embodiment, the invention encompasses a method
for treating a symptom of a disorder responsive to inhibition of sodium absorption across
an epithelial mucosal surface in a subject in need thereof, comprising administering a
peptide comprising a sequence selected from SEQ ID NOS:2-128 to the subject. The
disorder in the methods of the invention can be, in non-limiting examples, a lung
disorder (e.g., cystic fibrosis, non-cystic fibrosis bronchiectasis, chronic obstructive
pulmonary disease, acute or chronic bronchitis, or asthma), a gastrointestinal disorder
(e.g., inflammatory bowel disease), a kidney disorder, or a cardiovascular disorder.

[0074] Another aspect of the invention relates to a method of regulating salt
balance, blood volume, and/or blood pressure in a subject in need thereof, comprising
delivering to the subject a therapeutically effective amount of a specialized non-naturally
occurring peptide or a functional fragment or homolog thereof.

[0075] A third aspect of the invention relates to products that can be used to
carry out the methods disclosed herein. Thus, one aspect of the invention relates to a
peptide comprising the sequence:

\[ X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8 \ (SEQ \ ID \ NO:116) \]

wherein:

- \( X_1 \) is leucine or a conservative substitution with a natural or non-natural amino acid;
- \( X_2 \) is proline or a conservative substitution with a natural or non-natural amino acid;
- \( X_3 \) is valine or a conservative substitution with a natural or non-natural amino acid;
- \( X_4 \) is proline or a conservative substitution with a natural or non-natural amino acid;
- \( X_5 \) is leucine or a conservative substitution with a natural or non-natural amino acid;
- \( X_6 \) is aspartic acid or a conservative substitution with a natural or non-natural amino acid;
- \( X_7 \) is glutamine or a conservative substitution with a natural or non-natural amino acid; and
- \( X_8 \) is threonine or a conservative substitution with a natural or non-natural amino acid;
or a functional fragment thereof.

[0076] Another aspect of the invention relates to a peptide comprising the
sequence:

\[ X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8 \ (SEQ \ ID \ NO:117) \]
wherein:

- $X_1$ is leucine, norleucine, or valine;
- $X_2$ is proline, 4-hydroxyproline, (2R,5S)-5-phenyl-pyrrolidine-2-carboxylic acid, or 3,4-dehydro-L-proline;
- $X_3$ is valine, leucine, norleucine, or N-methylvaline;
- $X_4$ is proline, 4-hydroxyproline, (2R,5S)-5-phenyl-pyrrolidine-2-carboxylic acid, or 3,4-dehydro-L-proline;
- $X_5$ is leucine, norleucine, or valine;
- $X_6$ is aspartic acid or glutamic acid;
- $X_7$ is glutamine or asparagine; and
- $X_8$ is threonine, serine, or L-a-methylserine; or a functional fragment thereof.

[0077] A further aspect of the invention relates to a peptide comprising the sequence:

$$X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}$$

wherein $X_1$ is leucine or a conservative substitution with a natural or non-natural amino acid;
- $X_2$ is proline or a conservative substitution with a natural or non-natural amino acid;
- $X_3$ is valine or a conservative substitution with a natural or non-natural amino acid;
- $X_4$ is proline or a conservative substitution with a natural or non-natural amino acid;
- $X_5$ is leucine or a conservative substitution with a natural or non-natural amino acid;
- $X_6$ is aspartic acid or a conservative substitution with a natural or non-natural amino acid;
- $X_7$ is glutamine or a conservative substitution with a natural or non-natural amino acid;
- $X_8$ is threonine or a conservative substitution with a natural or non-natural amino acid;
- $X_9$ is threonine or a conservative substitution with a natural or non-natural amino acid;
- $X_{10}$ is leucine or a conservative substitution with a natural or non-natural amino acid;
- $X_{11}$ is proline or a conservative substitution with a natural or non-natural amino acid;
- $X_{12}$ is asparagine or a conservative substitution with a natural or non-natural amino acid;
- $X_{13}$ is valine or a conservative substitution with a natural or non-natural amino acid;
X₁₄ is asparagine or a conservative substitution with a natural or non-natural amino acid;
X₁₅ is proline or a conservative substitution with a natural or non-natural amino acid; or a functional fragment thereof.

[0078] In some embodiments, the peptide comprises a sequence selected from the group consisting of SEQ ID NOS:4-142 and SEQ ID NO:144. In one embodiment, the peptide comprises the sequence of SEQ ID NO:5 or SEQ ID NO:122. In one embodiment, the peptide comprises the sequence of SEQ ID NO:2 (aaLPVPLDQTLPLNVNpa) or SEQ ID NO:119. In one embodiment, the peptide comprises the sequence of SEQ ID NO:127 or SEQ ID NO:128 (aaPIPLDQTaa).

[0079] In certain embodiments, the peptides of the invention comprise at least one modified terminus, e.g., to protect the peptide against degradation. In some embodiments, the N-terminus is acetylated and/or the C-terminus is amidated. In some embodiments, the peptide comprises the sequence of any one of SEQ ID NOS:10-127, 129, 130, 133, 134, or 136-140, further comprising one or two D-alanines at the amino- and/or carboxyl-terminal ends.

[0080] In certain embodiments, the peptides of the invention comprise at least one non-natural amino acid (e.g., 1, 2, 3, or more) or at least one terminal modification (e.g., 1 or 2). In some embodiments, the peptide comprises at least one non-natural amino acid and at least one terminal modification.

[0081] In certain embodiments, the peptide mimics the sodium channel binding domain of a PLUNC protein. The sodium channel binding domain is the minimal fragment of the PLUNC protein required to have substantially the same binding activity to the sodium channel as the full length PLUNC protein. The term "substantially the same binding activity" refers to an activity that is at least about 50% of the binding activity of the full length protein, e.g., at least about 60%, 70%, 80%, or 90% of the binding activity. In some embodiments, the peptide has at least the same binding activity as the full length PLUNC protein. In one embodiment, the sodium channel is ENaC, e.g., human ENaC. In another embodiment, the sodium channel is one that is similar in sequence and/or structure to ENaC, such as acid-sensing ion channels (ASIC).

[0082] The peptides of the present invention can optionally be delivered in conjunction with other therapeutic agents. The additional therapeutic agents can be delivered concurrently with the peptides of the invention. As used herein, the word "concurrently" means sufficiently close in time to produce a combined effect (that is,
concurrently can be simultaneously, or it can be two or more events occurring within a short time period before or after each other. In one embodiment of the invention, the specialized non-naturally occurring peptide is delivered to a patient concurrently with a compound that modulates the function of the Cystic fibrosis transmembrane conductance regulator (CFTR) where the combined activity of the specialized non-naturally occurring peptide and the CFTR-targeted agent have superior activity to the CFTR-targeted agent alone. In another embodiment of the invention, the specialized non-naturally occurring peptide is delivered to a patient concurrently with a mucolytic compound where the combined activity of the specialized non-naturally occurring peptide and the mucolytic agent have superior activity to the mucolytic agent alone. In yet another embodiment of the invention, the specialized non-naturally occurring peptide is delivered to a patient concurrently with a long acting B-agonist compound (LABA) where the combined activity of the specialized non-naturally occurring peptide and the LABA agent have superior activity to the LABA alone. In yet another embodiment of the invention, the specialized non-naturally occurring peptide is delivered to a patient concurrently with a glucocorticoid agonist where the combined activity of the specialized non-naturally occurring peptide and the glucocorticoid agent have superior activity to the glucocorticoid alone.

[0083] Another aspect of the invention relates to a kit comprising the peptide of the invention and useful for carrying out the methods of the invention. The kit may further comprise additional reagents for carrying out the methods (e.g., buffers, containers, additional therapeutic agents) as well as instructions.

[0084] As a further aspect, the invention provides pharmaceutical formulations and methods of administering the same to achieve any of the therapeutic effects (e.g., modulation of sodium absorption) discussed above. The pharmaceutical formulation may comprise any of the reagents discussed above in a pharmaceutically acceptable carrier, e.g., a specialized non-naturally occurring peptide or functional fragment thereof.

[0085] By "pharmaceutically acceptable" it is meant a material that is not biologically or otherwise undesirable, i.e., the material can be administered to a subject without causing any undesirable biological effects such as toxicity.

[0086] The formulations of the invention can optionally comprise medicinal agents, pharmaceutical agents, carriers, adjuvants, dispersing agents, diluents, and the like.
[0087] The peptides of the invention can be formulated for administration in a pharmaceutical carrier in accordance with known techniques. See, e.g., Remington, *The Science And Practice of Pharmacy* (9th Ed. 1995). In the manufacture of a pharmaceutical formulation according to the invention, the peptide (including the physiologically acceptable salts thereof) is typically admixed with, *inter alia*, an acceptable carrier. The carrier can be a solid or a liquid, or both, and is preferably formulated with the peptide as a unit-dose formulation, for example, a tablet, which can contain from 0.01 or 0.5% to 95% or 99% by weight of the peptide. One or more peptides can be incorporated in the formulations of the invention, which can be prepared by any of the well-known techniques of pharmacy.

[0088] A further aspect of the invention is a method of treating subjects *in vivo*, comprising administering to a subject a pharmaceutical composition comprising a peptide of the invention in a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is administered in a therapeutically effective amount. Administration of the peptides of the present invention to a human subject or an animal in need thereof can be by any means known in the art for administering compounds.

[0089] The formulations of the invention include those suitable for oral, rectal, topical, buccal (e.g., sub-lingual), vaginal, parenteral (e.g., subcutaneous, intramuscular including skeletal muscle, cardiac muscle, diaphragm muscle and smooth muscle, intradermal, intravenous, intraperitoneal), topical (i.e., both skin and mucosal surfaces, including airway surfaces), intranasal, transdermal, intraarticular, intrathecal, and inhalation administration, administration to the liver by intraportal delivery, as well as direct organ injection (e.g., into the liver, into the brain for delivery to the central nervous system, into the pancreas, or into a tumor or the tissue surrounding a tumor). The most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular peptide which is being used.

[0090] For injection, the carrier will typically be a liquid, such as sterile pyrogen-free water, sterile normal saline, hypertonic saline, pyrogen-free phosphate-buffered saline solution, bacteriostatic water, or Cremophor EL[R] (BASF, Parsippany, N.J.). For other methods of administration, the carrier can be either solid or liquid.

[0091] For oral administration, the peptide can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. Peptides can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol,
starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that can be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, edible white ink and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance.

[0092] Formulations suitable for buccal (sub-lingual) administration include lozenges comprising the compound in a flavored base, usually sucrose and acacia or tragacanth; and pastilles comprising the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

[0093] Formulations of the present invention suitable for parenteral administration comprise sterile aqueous and non-aqueous injection solutions of the peptide, which preparations are preferably isotonic with the blood of the intended recipient. These preparations can contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions can include suspending agents and thickening agents. The formulations can be presented in unit/dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

[0094] Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously described. For example, in one aspect of the present invention, there is provided an injectable, stable, sterile composition comprising a peptide of the invention, in a unit dosage form in a sealed container. The peptide or salt is provided in the form of a lyophilizate which is capable of being reconstituted with a suitable pharmaceutically acceptable carrier to form a liquid composition suitable for injection thereof into a subject. The unit dosage form typically comprises from about 1 mg to about 10 grams of the peptide or salt. When the peptide or salt is substantially water-insoluble, a sufficient amount of
emulsifying agent which is pharmaceutically acceptable can be employed in sufficient quantity to emulsify the peptide or salt in an aqueous carrier. One such useful emulsifying agent is phosphatidyl choline.

[0095] Formulations suitable for rectal administration are preferably presented as unit dose suppositories. These can be prepared by admixing the peptide with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

[0096] Formulations suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which can be used include petroleum jelly, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

[0097] Formulations suitable for transdermal administration can be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration can also be delivered by iontophoresis (see, for example, Tyle, Pharm. Res. 3:318 (1986)) and typically take the form of an optionally buffered aqueous solution of the peptides. Suitable formulations comprise citrate or bis/tris buffer (pH 6) or ethanol/water and contain from 0.1 to 0.2M of the compound.

[0098] The peptide can alternatively be formulated for nasal administration or otherwise administered to the lungs of a subject by any suitable means, e.g., administered by an aerosol suspension of respirable particles comprising the peptide, which the subject inhales. The respirable particles can be liquid or solid. The term "aerosol" includes any gas-borne suspended phase, which is capable of being inhaled into the bronchioles or nasal passages. Specifically, aerosol includes a gas-borne suspension of droplets, as can be produced in a metered dose inhaler or nebulizer, or in a mist sprayer. Aerosol also includes a dry powder composition suspended in air or other carrier gas, which can be delivered by insufflation from an inhaler device, for example. See Ganderton & Jones, Drug Delivery to the Respiratory Tract, Ellis Horwood (1987); Gonda (1990) Critical Reviews in Therapeutic Drug Carrier Systems 6:273-313; and Raeburn et al, J. Pharmacol. Toxicol. Meth. 27:143 (1992). Aerosols of liquid particles comprising the peptide can be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. See, e.g., U.S. Patent No. 4,501,729. Aerosols of solid particles comprising the
peptide can likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

[0099] Alternatively, one can administer the peptide in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

[0100] Further, the present invention provides liposomal formulations of the peptides disclosed herein and salts thereof. The technology for forming liposomal suspensions is well known in the art. When the peptide or salt thereof is an aqueous-soluble salt, using conventional liposome technology, the same can be incorporated into lipid vesicles. In such an instance, due to the water solubility of the peptide or salt, the peptide or salt will be substantially entrained within the hydrophilic center or core of the liposomes. The lipid layer employed can be of any conventional composition and can either contain cholesterol or can be cholesterol-free. When the peptide or salt of interest is water-insoluble, again employing conventional liposome formation technology, the salt can be substantially entrained within the hydrophobic lipid bilayer which forms the structure of the liposome. In either instance, the liposomes which are produced can be reduced in size, as through the use of standard sonication and homogenization techniques.

[0101] The liposomal formulations containing the peptides disclosed herein or salts thereof, can be lyophilized to produce a lyophilizate which can be reconstituted with a pharmaceutically acceptable carrier, such as water, to regenerate a liposomal suspension.

[0102] In the case of water-insoluble peptides, a pharmaceutical composition can be prepared containing the water-insoluble peptide, such as for example, in an aqueous base emulsion. In such an instance, the composition will contain a sufficient amount of pharmaceutically acceptable emulsifying agent to emulsify the desired amount of the peptide. Particularly useful emulsifying agents include phosphatidyl cholines and lecithin.

[0103] In particular embodiments, the peptide is administered to the subject in a therapeutically effective amount, as that term is defined above. Dosages of pharmaceutically active peptides can be determined by methods known in the art, see, e.g., Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, Pa). The therapeutically effective dosage of any specific peptide will vary somewhat from peptide to peptide, and patient to patient, and will depend upon the condition of the patient and the route of delivery. As a general proposition, a dosage from about 0.1 to about 50
mg/kg will have therapeutic efficacy, with all weights being calculated based upon the weight of the peptide, including the cases where a salt is employed. Toxicity concerns at the higher level can restrict intravenous dosages to a lower level such as up to about 10 mg/kg, with all weights being calculated based upon the weight of the peptide, including the cases where a salt is employed. A dosage from about 10 mg/kg to about 50 mg/kg can be employed for oral administration. Typically, a dosage from about 0.5 mg/kg to 5 mg/kg can be employed for intramuscular injection. Particular dosages are about 1 µmol/kg to 50 µmol/kg, and more particularly to about 22 µmol/kg and to 33 µmol/kg of the peptide for intravenous or oral administration, respectively.

[0104] In particular embodiments of the invention, more than one administration (e.g., two, three, four, or more administrations) can be employed over a variety of time intervals (e.g., hourly, daily, weekly, monthly, etc.) to achieve therapeutic effects.

[0105] The present invention finds use in veterinary and medical applications. Suitable subjects include both avians and mammals, with mammals being preferred. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys, and pheasants. The term "mammal" as used herein includes, but is not limited to, humans, bovines, ovines, caprines, equines, felines, canines, lagomorphs, etc. Human subjects include neonates, infants, juveniles, and adults.

[0106] The present invention is more particularly described in the following examples that are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

**EXAMPLE 1**

**Experimental Methods**

[0107] **Tissue procurement and cell culture:** Cells were harvested by enzymatic digestion from human bronchial tissue as previously described under a protocol approved by the UNC School of Medicine IRB (Tarran et al., J. Gen. Physiol. 127:591 (2006)). Human excess donor lungs and excised recipient lungs were obtained at the time of lung transplantation from portions of main stem or lumbar bronchi and cells were harvested by enzymatic digestion. All preparations were maintained at an air-liquid interface in a modified bronchial epithelial medium and used 2-5 weeks after seeding on 12 mm T-Clear inserts (Corning Costar) coated with human placental type VI
collagen (Sigma). Phosphate buffered saline (PBS) was used for washing human bronchial epithelial culture mucosal surfaces.

[0108] **Confocal microscopy:** To label airway surface liquid, Ringer containing Texas Red-dextran (2 mg/ml; Invitrogen) was added to human bronchial epithelial culture mucosal surfaces. Perfluorocarbon was added mucosally to prevent evaporation of the airway surface liquid and the culture placed in a chamber containing 100 μl Ringer on the stage of a Leica SP8 confocal microscope with a 63x glycerol immersion objective. 10 points per culture were scanned and an average airway surface liquid height determined. For confocal microscopy human bronchial epithelial cultures were bathed serosally in a modified Ringer solution containing (mM): 116 NaCl, 10 NaHCO$_3$, 5.1 KCl, 1.2 CaCl$_2$, 1.2 MgCl$_2$, 20 TES, 10 glucose, pH 7.4). At all other times, human bronchial epithelial cultures were maintained in a modified BEGM growth medium which contained 24 mM NaHCO$_3$ gassed with 5% CO$_2$. Perfluorocarbon (FC-77) was obtained from 3M and had no effect on ASL height as previously reported.

[0109] **Internalization of alpha-ENaC:** HEK293T cells were transfected with gfp-ocENaC where gfp was fused at the N-terminus of ENaC and unlabeled pENaC and yENaC using lipofectamine in 384 well plates as per the manufacturer's instructions and as published previously (see, e.g., Hobbs et al. Am J Physiol Lung Cell Mol Physiol. 2013 Dec;305(12):L990-L1001. doi: 10.1152/ajplung.00103.2013. Epub 2013 Oct; Garland et al. Proc Natl Acad Sci U S A. 2013 Oct 1;110(40):15973-8. doi: 10.1073/pnas.131999110. Epub 2013 Sep 16.; Tan et al. J Physiol. 2014 Dec 1;592(Pt 23):5251-68). Twenty-four hours later, peptide or vehicle control were added at t=0 and fluorescence was read using a Tecan M1000 plate reader 3 h later. Fluorescence was read at 488 and 510 nm.

[0110] Fig. 6 shows the results of an experiment analyzing the effects of various peptides on internalization of alpha-ENaC in HEK293T cells when GFP-tagged alpha-ENaC is co-expressed only with gamma ENaC and no beta-ENaC. In this figure, SEQ ID NO: 143 (negative control peptide) and water (vehicle) controls are used along with S18 (SEQ ID NO:1, and SEQ ID NO: 128. While SEQ ID NO:1 AND SEQ ID NO: 128 are effective in reducing alpha-ENaC when beta-ENaC is co-expressed, no effect is observed in this experiment when beta-ENaC is not present.

[0111] **Efficacy testing in pENaC mice:** Like CF patients, pENaC-Tg mice are disease free at birth, but soon develop obstructive lung disease (Zhou, Z., et al. Am J
Respir Crit Care Med 178, 1245-1256 (2008)). C57BL:FVB and C57BL:C3H mixed strains have 90% and 50% mortality, respectively, and the reproducibility of the disease on these backgrounds is sufficiently high that reliable data are produced with n of about 8-10 animals/group. Furthermore, the pENaC-Tg mouse responds to therapeutic interventions in a fashion similar to CF/COPD in humans, and the development of lung disease can be prevented following inhibition of lung disease at birth (Livraghi, A., et al. J Immunol 182, 4357-4367 (2009)). S18-derived peptides or vehicle were dosed one to three times a day by intranasal instillation (1μl/g body weight) for 14 days in parallel cohorts of mice. Mice were weighed daily and, if a diuretic effect was found, the volume excreted was replaced by sub-cutaneous injections of sterile saline. At the end of the treatment, mice were sacrificed for phenotypic analysis.

**[0112] Statistical analyses:*** All data are presented as the mean ± SE for n experiments. Airway cultures derived from three or more separate donors were used for each study and each oocyte study was repeated on three separate occasions. Differences between means were tested for statistical significance using paired or unpaired t tests or their non-parametric equivalent as appropriate to the experiment. From such comparisons, differences yielding P ≤ 0.05 were judged to be significant. All binding assays were fitted to the Hill equation.

**EXAMPLE 2**

**Identification of specialized non-naturally occurring peptides**

**[0113]** Based on the ability of normal human bronchial epithelial cultures to regulate airway surface liquid height to 7 μη, which was paralleled by a decrease in trypsin-sensitive ENaC activity, we previously demonstrated that SPLUNC1 derived peptides (namely, S18; FIG. 1) can inhibit ENaC with EC50 in the sub micromolar range for up to 24 h following a single dose (Hobbs et al, 2013). While S18 is resistant to proteolysis and heat-stable, we tested whether we could reduce the size of S18, increase its stability and/or increase its potency. Any of these actions would increase its utility as a drug.

**[0114]** To confirm our alanine-scan data, we made a peptide of subsequence A (LPVPLDQT (SEQ ID NO:1 13); FIG. 1). As a control, we made an overlapping 2nd peptide, which contained the charged mid-region (DQT), as well as subsequence B (DQTLPLNVNP (SEQ ID NO: 114)). As shown in FIG. 2, LPVPLDQT (SEQ ID
NO: 113) retained similar activity to S18, whilst DQTLPLNVNP (SEQ ID NO: 114) was inactive. However, additional experiments, where we examined susceptibility to proteases, demonstrated that whilst LPVPLDQT (SEQ ID NO: 113) could inhibit ENaC-led fluid absorption, this peptide was more susceptible to proteolytic degradation. Thus, we generated new peptides where we flanked the C- and N- termini with a pair of D-alanines (shown as lower case 'a'). Peptides were added mucosally to HBECs at 30 µM and ASL height measured 3 h later by XZ-confocal microscopy. FIG. 2A shows the comparison of the N-terminal region of S18 (LPVPLDQT) (SEQ ID NO:1 13) vs. an overlapping, but C-terminal segment (DQTLPLNVNP) (SEQ ID NO:1 14). In FIG. 2B, D-alanines were added to Sub-S18 peptides LPVPLDQT (SEQ IDNO:113) and LPVPLDQTLP (SEQ ID NO: 115) to increase the stability of LPVPLDQT (SEQ ID NO: 113). D-alanines are noted as lowercase a. All n = 6-9. Data are shown as mean ± SEM.

[0115] The 15-mer and 8-mer showed identical activity when compared against S18 (FIGS. 2A-2B). We then generated full dose-responses for S18 vs. the peptide aaLPVPLDQTaa (SEQ ID NO:3). S18 and aaLPVPLDQTaa (SEQ ID NO:3) produced identical dose responses (FIG. 3A), indicating that shortening the peptide and flanking it with unnatural amino acids did not affect its potency or efficacy. Next, we made several new peptides based on the sequence of SEQ ID NO:2. Here, since prolines typically provide kinks in a peptide, they were not altered and instead, we systematically made conservative substitutions of the other amino acids. The dose responses are shown in FIG. 3B. Note, since there are several lines on this graph, the error bars and data symbols were omitted. However, these data were obtained simultaneously using paired airway cultures, so their comparison is valid. Clearly, SEQ ID NO: 5 (aaLPNlePLDQTaa) shows a log-fold increase in potency as compared to S18, whilst SEQ ID NO: 9 (aaLPVPLDQSaa) and SEQ ID NO:6 (aaLPVPNleDQQTaa) show diminished potency and efficacy respectively (FIG. 3B). Other sequences included in FIG. 3B are SEQ ID NO: 4 (aaNlePVPLDQTaa), SEQ ID NO: 7 (aaLPVPLDNTaa) and SEQ ID NO: 8 (aaLPVPLEQTaa).

[0116] Using a fresh batch of airway cells, we performed an additional dose response to SEQ ID NO: 5 (FIG. 4). As can be seen, this peptide gave a significant increase in potency, as compared to S18, and the IC₅₀ was 30 nM.
The ability of the peptides to modulate internalization of αENaC was tested. Results from these experiments are shown in FIGS. 5A-5U. ADG is a negative control peptide with the sequence: NH$_3$-ADGGLLLLNNPPPPQTV V-NH$_2$ (SEQ ID NO: 143). The peptides used in these experiments were S18 (SEQ ID NO: 1), SEQ ID NO:2, SEQ ID NO:141, SEQ ID NO:129, SEQ ID NO:130, SEQ ID NO:128, SEQ ID NO: 131, SEQ ID NO:132, SEQ ID NO: 133, SEQ ID NO:134, SEQ ID NO:135, SEQ ID NO:136, SEQ ID NO:137, SEQ ID NO: 138, SEQ ID NO: 131, SEQ ID NO:139; SEQ ID NO: 140, SEQ ID NO: 144 and SEQ ID NO: 127. Also used in these experiments were peptides of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:136, each with an aa cap at both the N- and the C-terminus. Also shown is amiloride, which inhibits ENaC by another mechanism and does not reduce the amount of functional receptor on the surface of cells. These results demonstrate that the SPLUNC peptides trigger the destruction of the ENaC function to inhibit its activity.

The efficacy of the peptides was tested in pENaC mice. Results from these experiments are shown in Figs. 7A-7F. Fourteen-day treatment with inhaled SEQ ID NO: 128 was more effective than inhaled SEQ ID NO: 142 at increasing percent survival of pENaC-Tg C57BL:FVB mice (Fig. 7B). Fourteen-day treatment with SEQ ID NO:128 also increased percent survival of pENaC-Tg C57BL:C3H mice (Figs. 7C and 7D). Once daily dosing with SEQ ID NO: 128 increased percent survival of pENaC-Tg C57BL:C3H mice (Fig. 7E, left panel). The right panel of Fig. 7E shows that SEQ ID NO: 128 did not have a diuretic effect. Fig. 7F shows that treatment with SEQ ID NO:127 increased percent survival of ENaC-Tg C57BL:C3H mice. Sequences of other peptides used in the experiment were SEQ ID NO: 129, SEQ ID NO: 134, and SEQ ID NO:136.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.
That which is claimed is:

1. A peptide comprising the sequence:

   \[ X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8 \] (SEQ ID NO: 116)

   wherein:
   - \( X_1 \) is leucine or a conservative substitution with a natural or non-natural amino acid;
   - \( X_2 \) is proline or a conservative substitution with a natural or non-natural amino acid;
   - \( X_3 \) is valine or a conservative substitution with a natural or non-natural amino acid;
   - \( X_4 \) is proline or a conservative substitution with a natural or non-natural amino acid;
   - \( X_5 \) is leucine or a conservative substitution with a natural or non-natural amino acid;
   - \( X_6 \) is aspartic acid or a conservative substitution with a natural or non-natural amino acid;
   - \( X_7 \) is glutamine or a conservative substitution with a natural or non-natural amino acid; and
   - \( X_8 \) is threonine or a conservative substitution with a natural or non-natural amino acid;

   or a functional fragment thereof.

2. A peptide comprising the sequence:

   \[ X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12} \] (SEQ ID NO: 117)

   wherein:
   - \( X_1 \) is leucine, norleucine, or valine;
   - \( X_2 \) is proline, 4-hydroxyproline, (2R,5S)-5-phenyl-pyrrolidine-2-carboxylic acid, or 3,4-dehydro-L-proline;
   - \( X_3 \) is valine, leucine, norleucine, or N-methylvaline;
   - \( X_4 \) is proline, 4-hydroxyproline, (2R,5S)-5-phenyl-pyrrolidine-2-carboxylic acid, or 3,4-dehydro-L-proline;
   - \( X_5 \) is leucine, norleucine, or valine;
   - \( X_6 \) is aspartic acid or glutamic acid;
   - \( X_7 \) is glutamine or asparagine; and
   - \( X_8 \) is threonine, serine, or L-a-methylserine;

   or a functional fragment thereof.
3. The peptide of claim 1 or 2, comprising the sequence of any one of SEQ ID NOS:4-115.

4. A peptide comprising the sequence:

\[ \text{X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15} \] (SEQ ID NO: 118)

wherein:

- \( X_1 \) is leucine or a conservative substitution with a natural or non-natural amino acid;
- \( X_2 \) is proline or a conservative substitution with a natural or non-natural amino acid;
- \( X_3 \) is valine or a conservative substitution with a natural or non-natural amino acid;
- \( X_4 \) is proline or a conservative substitution with a natural or non-natural amino acid;
- \( X_5 \) is leucine or a conservative substitution with a natural or non-natural amino acid;
- \( X_6 \) is aspartic acid or a conservative substitution with a natural or non-natural amino acid;
- \( X_7 \) is glutamine or a conservative substitution with a natural or non-natural amino acid;
- \( X_8 \) is threonine or a conservative substitution with a natural or non-natural amino acid;
- \( X_9 \) is threonine or a conservative substitution with a natural or non-natural amino acid;
- \( X_{10} \) is leucine or a conservative substitution with a natural or non-natural amino acid;
- \( X_{11} \) is proline or a conservative substitution with a natural or non-natural amino acid;
- \( X_{12} \) is asparagine or a conservative substitution with a natural or non-natural amino acid;
- \( X_{13} \) is valine or a conservative substitution with a natural or non-natural amino acid;
- \( X_{14} \) is asparagine or a conservative substitution with a natural or non-natural amino acid; and
- \( X_{15} \) is proline or a conservative substitution with a natural or non-natural amino acid; or a functional fragment thereof.

5. The peptide of claim 4, comprising the sequence of SEQ ID NO: 120.

6. A peptide comprising the sequence of SEQ ID NO:127 or SEQ ID NO:128.
7. The peptide of any one of claims 1-6, wherein the N-terminus is acetylated and/or the C-terminus is amidated.

8. The peptide of any one of claims 1-7, wherein the peptide comprises at least one non-natural amino acid or at least one terminal modification.

9. The peptide of any one of claims 1-7, further comprising at least one D-alanine at the N-terminus and/or the C-terminus.

10. A kit comprising the peptide of any one of claims 1-9.

11. A method of inhibiting the activation of a sodium channel, comprising contacting a sodium channel with the peptide of any one of claims 1-9.

12. The method of claim 11, wherein the sodium channel is an epithelial sodium channel (ENaC).

13. The method of claim 11, wherein the sodium channel is present in an isolated cell.

14. The method of claim 13, wherein the isolated cell is part of an epithelial cell culture.

15. The method of claim 11, wherein the sodium channel is present in a cell in an animal.

16. The method of claim 15, wherein the animal is a disease model.

17. The method of claim 11, wherein contacting the sodium channel with the peptide comprises delivering the peptide to a cell comprising the sodium channel.

18. The method of claim 11, wherein activation of the sodium channel is inhibited by at least 20%.
19. The method of claim 11, wherein activation of the sodium channel is inhibited by at least 50%.

20. The method of claim 11, wherein activation of the sodium channel is inhibited by at least 90%.

21. A method of inhibiting sodium absorption through a sodium channel, comprising contacting the sodium channel with the peptide of any one of claims 1-9.

22. The method of claim 21, wherein the sodium channel is ENaC.

23. A method of increasing the volume of fluid lining an epithelial mucosal surface, comprising contacting a sodium channel present on the epithelial mucosal surface with the peptide of any one of claims 1-9.

24. The method of claim 23, wherein the sodium channel is ENaC.

25. A method of reducing the level of a sodium channel present on the surface of a cell, comprising contacting the sodium channel with the peptide of any one of claims 1-9.

26. The method of claim 25, wherein the sodium channel is ENaC.

27. A method of treating a disorder responsive to inhibition of sodium absorption across an epithelial mucosal surface in a subject in need thereof, comprising delivering to the subject a therapeutically effective amount of the peptide of any one of claims 1-9.

28. The method of claim 27, wherein the sodium channel is ENaC.

29. The method of claim 27, wherein said disorder is a lung disorder.

30. The method of claim 27, wherein said lung disorder is cystic fibrosis.
31. The method of claim 27, wherein said lung disorder is non-cystic fibrosis bronchiectasis.

32. The method of claim 27, wherein said lung disorder is chronic obstructive pulmonary disease.

33. The method of claim 27, wherein said lung disorder is acute or chronic bronchitis.

34. The method of claim 27, wherein said lung disorder is asthma.

35. The method of claim 27, wherein said disorder is a gastrointestinal disorder.

36. The method of claim 35, wherein said gastrointestinal disorder is inflammatory bowel disease.

37. The method of claim 27, wherein said disorder is a kidney disorder.

38. A method of regulating salt balance, blood volume, and/or blood pressure in a subject in need thereof, comprising delivering to the subject a therapeutically effective amount of the peptide of any one of claims 1-9.

39. The method of claim 27, wherein delivering the peptide reduces the level of a sodium channel present on the surface of a cell in the subject.

40. A method for treating a symptom of a lung disorder, a gastrointestinal disorder, a kidney disorder, or a cardiovascular disorder in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the peptide of any one of claims 1-9.

41. The method of claim 40, wherein the lung disorder is cystic fibrosis, non-cystic fibrosis bronchiectasis, chronic obstructive pulmonary disease, acute or chronic bronchitis, or asthma.
42. The method of claim 40, wherein the gastrointestinal disorder is inflammatory bowel disease.
GGL\textsuperscript{24}PVPLDQT\textsuperscript{31}LPLNVNP\textsuperscript{38}A

\textbf{FIG. 1}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2a}
\caption{FIG. 2A}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2b}
\caption{FIG. 2B}
\end{figure}
FIG. 4
FIG. 5B

GFP-α/βγ ENaC
t=3 hr
HEK 293T

Fluorescence units / well

-16 -14 -13 -12 -11 -10 -9 -8 -7 -6

log [drug]

SEQ ID NO: 141
SEQ ID NO: 129
SEQ ID NO: 128

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EC50
Std. Error
LogEC50

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-9.067
-12.27
-12.77
-10.79
-12.36
-11.87
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8.562e-010
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1.699e-013
1.635e-011
4.342e-013
1.363e-012
0.1462
0.1103
0.1120
0.1329
0.1487
0.1813
0.2072
FIG. 5C

GFP-αβγ ENaC

t=3 hr

HEK 293T

Fluorescence units / well

log [drug]

SEQ ID NO:130
SEQ ID NO:128
SEQ ID NO:131

LogEC50
EC50
Std. Error
LogEC50

-9.138
7.270e-010
0.1462

-9.067
8.562e-010
0.1103

-12.27
5.355e-013
0.1120

-12.77
1.699e-013
0.1329

-10.79
1.635e-011
0.1487

-12.36
4.342e-013
0.1813

-11.87
1.363e-012
0.2072
FIG. 5E

GFP-α/βγ ENaC

* t=3 hr

HEK 293T

- SEQ ID NO:127
- SEQ ID NO:137
- SEQ ID NO:128

Fluorescence units / well

log [drug]

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FIG. 5F

GFP-α/βγ ENaC
t=3 hr
HEK 293T

Fluorescence units/well vs log [drug]

SEQ ID NO: 138
SEQ ID NO: 131
SEQ ID NO: 128

LogEC50
EC50
Std. Err
LogEC50
0.1526 0.1134 0.1619 0.1604 0.1147 0.1586 0.1415 0.09955 0.5554
FIG. 5H

GFP-α/βγ ENaC

t=3 hr

HEK 293T

Fluorescence units / well

log [drug]

-15 -14 -13 -12 -11 -10 -9 -8 -7 -6

SEQ ID NO:140

SEQ ID NO:128

SEQ ID
NO:140
-11.93

SEQ ID
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-12.36

SEQ ID
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-10.30

SEQ ID
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-10.66

SEQ ID
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-12.02

SEQ ID
NO:16
-10.74

SEQ ID
NO:17
-10.84

SEQ ID
NO:18
-11.66

SEQ ID
NO:144
-12.15

LogEC50
1.165e-012

EC50

Std. Error
0.2067

0.09955 0.2428 0.1998 0.1572 0.1753 0.1430 0.1318 0.1446

**GFP-α/βγ ENaC**

**t=3 hr**

**HEK 293T**

- SEQ ID NO:128
- SEQ ID NO:136
- SEQ ID NO:12

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Fluorescence units / well

log [drug]
FIG. 5J

GFP-α/βγ ENaC
t=3 hr
HEK 293T

Fluorescence units / well

log [drug]

SEQ ID NO: 128
SEQ ID NO: 13
SEQ ID NO: 16

LogEC50
-11.93
1.156e-012

EC50

Std. Error

LogEC50
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FIG. 5L

GFP-α/βγ ENaC
t=3 hr
HEK 293T

Fluorescence units / well

log [drug]

SEQ ID NO: 128
SEQ ID NO: 144

LogEC50
EC50
Std. Error
LogEC50

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Cell viability
HEK 293T
GFP-α/βγ ENaC

Fluorescence units / well

log [drug]

-15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3

SEQ ID NO: 130
SEQ ID NO: 128
SEQ ID NO: 131
FIG. 5P

Cell viability
HEK 293T
GFP-α/βγ ENaC

Fluorescence units / well

log [drug]

-15 -14 -13 -12 -11 -10 -9 -8 -7 -6

• SEQ ID NO: 133
• SEQ ID NO: 135
• SEQ ID NO: 128
FIG. 5Q

Cell viability
HEK 293T
GFP-α/β ENaC

Fluorescence units / Well

log [drug]

55000 -
50000 -
45000 -
40000 -
35000 -
30000 -
25000 -
-5 15 -14 -13 -12 -11 -10 -9 -8 -7 -6
Cell viability
HEK 293T
GFP-\(\alpha/\beta\gamma\) ENaC

Fluorescence units / well

log [drug]

-15 -14 -13 -12 -11 -10 -9 -8 -7 -6

- 55000 - 50000 - 45000 - 40000 - 35000 - 30000 - 25000

○ SEQ ID NO: 138
□ SEQ ID NO: 131
▲ SEQ ID NO: 139
● SEQ ID NO: 128
FIG. 5T

Cell viability
HEK 293T
GFP-α/βγ ENaC

Fluorescence units / well

-15 -14 -13 -12 -11 -10 -9 -8 -7 -6
log [drug]

● SEQ ID NO:136
■ SEQ ID NO:12
▲ SEQ ID NO:13
Cell viability
HEK 293T
GFP-\alpha/\beta ENaC

Fluorescence units / well

log [drug]
FIG. 7C

SPY3: C57/C3H βENaC Mouse Treatment

Legend:
- ▲ SEQ ID NO: 128 @ 3 mM
- ■ SEQ ID NO: 128 @ 30 mM
- ● SEQ ID NO: 129 @ 3mM
- ○ NaCl

Graph showing percent survival over days elapsed.
FIG. 7F

Survival proportions

- 50mM SEQ ID NO:127 (n=3)
- 50mM SEQ ID NO:136 (n=5)
- NaCl n=6
- 50mM SEQ ID NO:134 (n=6)
**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US 2015/054693

### A. CLASSIFICATION OF SUBJECT MATTER

(see extra sheet)

According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/04, 38/16, A61P 1/00, 11/06, 11/08, 11/12, C07K14/435

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>X</td>
<td>WO 2013/043720 A1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL) 28.03.2013, paragraph [0098], claims</td>
<td>1-7, 10-42</td>
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<td>WO 2009/074575 A2 (NOVARTIS AG et al.) 18.06.2009, claims</td>
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* Special categories of cited documents:  
  "A" document defining the general state of the art which is not considered to be of particular relevance  
  "E" earlier document published on or after the international filing date  
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
  "O" document referring to an oral disclosure, use, exhibition or other means  
  "P" document published prior to the international filing date but later than the priority date claimed  
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  
  "&" document member of the same patent family

Date of the actual completion of the international search: 28 December 2015 (28.12.2015)

Date of mailing of the international search report: 21 January 2016 (21.01.2016)

Name and mailing address of the ISA/RU:  
Federal Institute of Industrial Property,  
Berezhkovskaya nab., 30-1, Moscow, G-59,  
GSP-3, Russia, 125993  
Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37  
Authorized officer: O. Plekhotkina  
Telephone No. 495 531 65 15

Form PCT/ISA/210 (second sheet) (January 2015)
Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-9  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.
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