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(54) **Title**: MUCOLYTIC COMPOSITIONS AND USES THEREOF

(57) **Abstract**: Mucolytic compositions comprising short peptides are provided, and methods for promoting mucus clearance and treating diseases or disorders where mucus is accumulated or disturbing breathing.

MUCOLYTIC COMPOSITIONS AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to mucolytic compositions comprising short peptides, and uses thereof for promoting clearance of mucus and as mucokinetic agents.

BACKGROUND OF THE INVENTION

Mucus is a viscous secretion produced by, and covering, the epithelial lining of body cavities that communicate with the external environment, including the respiratory, gastrointestinal and urogenital systems in mammals. It is produced by specialized epithelial cells known as goblet cells, and sub-mucosal glands. The main function of mucus is to lubricate and protect these epithelial surfaces against infectious agents such as fungi, bacteria and viruses.

In the respiratory system, mucus aids in the protection of the lungs by trapping inhaled foreign particles. Motile cilia underlying the mucus layer advance the mucus containing the trapped particles towards the oropharynx, where it is either expelled from the body by expectoration (as sputum), or swallowed and destroyed by the digestive system. This mucociliary clearance mechanism serves to keep sterility of the lower respiratory tract and prevent mucus accumulation in the lungs.

Mucus is in effect a heterogeneous mixture containing water, salts and various macromolecules. The main polymeric components of mucus are densely glycosylated glycroproteins known as mucins. Mucins are produced and secreted by goblet cells.

Mucins are typically secreted as large aggregates in which mucin monomers are linked to one another by covalent and non-covalent interactions. Exemplary interactions include intramolecular disulfide bonds between cystein residues, hydrogen bonds between sugar units, and ionic interactions between positively and negatively charged amino acid residues and sugar units.

Mucin hyper-secretion and/or impaired mucus clearance from the lungs and airways are common features of a number of respiratory diseases, including for example, bronchitis, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), sinusitis and pneumonia. Products of inflammation, including leukocytes-derived DNA and filamentous actin (F-actin), effete cells, bacteria, and cell debris may alter the physical properties of mucus and contribute to mucus purulence, thickness and viscosity in these

conditions. Impaired mucus clearance is also associated with, *inter alia*, continued exposure to cigarette smoke or atmospheric pollutants.

Mucokinetics are a class of drugs that aid in the clearance of respiratory tract secretions, typically administered by inhalation or oral routes. Examples include: hypoviscosity agents, such as isotonic or hypotonic saline solutions that decrease mucus viscosity by increasing its water content through osmotic effects; expectorants (bronchomucotropics), such as guaiphenesin that facilitates mucus expectoration by reducing adhesiveness and surface tension of mucus, and increasing bronchial secretion and ciliary action; and mucolytics that decrease mucus viscosity by disrupting cross-links within the mucus gel structure, and by separating between the mucus and the white blood cells that the mucus may contain. The latter includes, for example: N-acetylcysteine (NAC) and carbocisteine, which break disulfide bonds between mucoproteins inclusive of mucin monomers; hypertonic saline, which breaks ionic bonds; and bromhexine, which disrupts the structure of mucopolysaccharides. The group of mucolytics also includes drugs that degrade DNA, fibrin, or F-actin in airway secretions, for example, dornase alpha, which is a solution of recombinant human deoxyribonuclease I (rhDNase).

The known medications have several drawbacks. For example, saline solutions may cause bronchospasm and hypernatremia, some expectorants are known to cause gastric irritation, mucolytic agents such as NAC are characterized by unpalatable taste and smell and associated with adverse effects such as nausea, vomiting, stomatitis and rhinorrhea, and the dornase alpha DNAse solution has a delayed onset of action of 3-7 days in addition to some undesired side effects.

WO 2002/034202 discloses an antioxidant compound characterized by (a) a peptide including at least three amino acid residues of which at least two are cysteine residues, each having a readily oxidizable sulfhydryl group for effecting antioxidation; and at least two peptide bonds, each being cleavable by at least one intracellular peptidase; and (b) a first hydrophobic or non-charged moiety being attached to an amino terminal of the peptide via a first bond and a second hydrophobic or non-charged moiety being attached to a carboxy terminal of the peptide via a second bond, the first hydrophobic or non-charged moiety and the second hydrophobic or non-charged moiety are selected so as to provide the antioxidant compound with membrane miscibility properties for permitting the antioxidant compound to cross cellular membranes; wherein cleavage of the at least two peptide bonds by the at least one intracellular peptidase results in generation of a plurality

of antioxidant species, each including one of the cysteine residues having the readily oxidizable sulfhydryl group and which is also active in effecting antioxidation, thereby providing for a plurality of different antioxidant species acting in synergy in exerting antioxidation.

WO 2012/098546, to the inventor of the present invention and others, discloses potent compounds having combined antioxidant, anti-inflammatory, anti-radiation and metal chelating properties. Short peptides having said properties and methods and uses of such short peptides in clinical and cosmetic applications are disclosed. Among other peptides, Cys-Lys-Met-Cys (SEQ ID NO: 1) and Cys-Met-Lys-Cys (SEQ ID NO: 2) are disclosed.

WO 2014/016837, to the inventor of the present invention, discloses compositions and methods utilizing thiol-containing short peptides having the sequence Cys-Lys-Met-Cys (SEQ ID NO: 1) and optionally N- and C- terminal modifications, for increasing carnitine level in muscle tissues, and treating or preventing diseases or disorders affecting muscle tissue.

WO 2014/016831, to the inventor of the present invention, discloses compositions and methods for treating blood disorders associated with glutathione dysregulation, utilizing short thiol-containing peptides selected, *inter alia*, from Cys-Lys-Met-Cys (SEQ ID NO: 1) and Cys-Met-Lys-Cys (SEQ ID NO: 2), optionally with N- and C- terminal modifications. Further disclosed are compositions and methods for preserving biological samples using the peptides.

Improved compositions and methods for reducing viscosity of respiratory secretions to facilitate their clearance are needed. Such compositions and methods may be useful, for example, in the treatment of diseases where excess mucus is present in the respiratory tract.

SUMMARY OF THE INVENTION

The present invention according to some aspects provides potent mucolytic compositions comprising short peptides. The peptide compounds utilized herein comprise lysine and methionine amino acid residues located between two cysteine residues. In some embodiments, the peptides further comprise N- and C- terminal modifications, such as N- and C- terminal blocking groups.

According to further aspects the present invention further provides methods for promoting mucus clearance, and methods for treating diseases or disorders where mucus is accumulated in the respiratory tract, utilizing the above compositions.

The peptides utilized herein were found to be highly effective in reducing the viscosity of sputum samples collected from patients. Surprisingly, the peptides were able to lower the viscosity and liquefy the sputum samples completely, much more effectively than N-acetylcysteine (NAC) and at significantly lower concentrations. NAC is widely used as a mucolytic drug (through inhalation and oral administration routes). Advantageously, the peptides showed complete liquefaction of the sputum samples within a shorter period of time compared to NAC. Rapid effect of a mucolytic agent may be particularly beneficial, for example, for inhalation administration, in cases where several drugs are administered concurrently. It is contemplated that rapid clearance of mucus from the airways would facilitate better absorption and more effective activity of the co-administered drugs.

According to one aspect, the present invention provides a method for promoting mucus clearance in a subject in need thereof, the method comprising administering to the subject a mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1); and

Cys-Met-Lys-Cys (SEQ ID NO: 2).

According to another aspect, the present invention provides a mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1); and

Cys-Met-Lys-Cys (SEQ ID NO: 2)

for use in promoting mucus clearance.

In some embodiments, the method and composition are used for the treatment of a disease or disorder where airway mucus is accumulated or disturbing breathing.

According to another aspect, the present invention provides a method for treating a disease or disorder where airway mucus is accumulated or disturbing breathing, the method comprising administering to a subject in need thereof a mucolytic pharmaceutical

composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1); and

Cys-Met-Lys-Cys (SEQ ID NO: 2).

According to yet another aspect, the present invention provides a mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1); and

Cys-Met-Lys-Cys (SEQ ID NO: 2)

for use in the treatment of a disease or disorder where airway mucus is accumulated or disturbing breathing.

In some embodiments, the disease or disorder is an infectious respiratory disease (including bacterial, fungal or viral). In some embodiments, the infectious respiratory disease is selected form the group consisting of acute sinusitis, chronic sinusitis, acute bronchitis, bronchiolitis, pneumonia and tuberculosis. Each possibility represents a separate embodiment of the invention.

In some embodiments, the disease or disorder is a degenerative lung disease. In some embodiments, the degenerative lung disease is selected form the group consisting of chronic obstructive pulmonary disease (COPD), emphysema, bronchiectasis and chronic bronchitis. Each possibility represents a separate embodiment of the invention.

In some embodiments, the disease or disorder is a genetic disease. In some embodiments, the genetic disease is cystic fibrosis (CF). In other embodiments, the genetic disease is ciliary dyskinesia (primary or secondary). Each possibility represents a separate embodiment of the invention.

In some embodiments, the disease or disorder is an interstitial lung disease. In some embodiments, the interstitial lung disease is a pulmonary fibrosis, such as idiopathic pulmonary fibrosis.

In some embodiments, the disease or disorder is lung inflammation.

In some preferred embodiments, the compositions of the present invention are formulated for local administration. In some embodiments, they are formulated for inhalation administration. In additional embodiments, they are formulated for nasal administration.

In some embodiments, the peptide present in the compositions of the present invention further comprises at least one modification of the peptide's terminus. According to some embodiments, the peptide comprises an amino-terminal modification. According to other embodiments, the peptide comprises a carboxy-terminal modification. According to yet other embodiments, the peptide comprises both amino-terminal and carboxy-terminal modifications. Each possibility represents a separate embodiment of the invention.

In principle, any group suitable for amino terminus modification, and any group suitable for carboxy terminus modification may be used for the peptide used according to embodiments of the present invention.

In some embodiments, the amino terminal modification is an amino terminal blocking group.

In some typical embodiments, the amino-terminal blocking group is selected from the group consisting of alkyl and acyl. Each possibility represents a separate embodiment of the invention.

In some exemplary embodiments, the amino-terminal blocking group is an acetyl group.

In some embodiments, the amino terminal modification is a moiety that improves the ability of the peptide to penetrate lipid layers. In some exemplary embodiments, the moiety that improves that ability of the peptide to penetrate lipid layers is a fatty acid. In some embodiments, the fatty acid is selected from the group consisting of palmitic acid, phosphatidic acid, stearic acid, arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid, and oleic acid. Each possibility represents a separate embodiment of the invention.

In some embodiments, the amino terminal modification is selected from the group consisting of an amino terminal blocking group and a fatty acid. Each possibility represents a separate embodiment of the invention.

In some embodiments, the amino terminal modification is selected from the group consisting of alkyl, acyl and a fatty acid.

In some embodiments, the carboxy terminal modification is a carboxy terminal blocking group. In some typical embodiments, the carboxy terminal blocking group is selected from the group consisting of amide, ester and alcohol group. Each possibility represents a separate embodiment of the invention. In some exemplary embodiments, the carboxy terminal blocking group is an amide group.

In some exemplary embodiments, the compositions comprise the peptide N-acetyl-Cys-Lys-Met-Cys-amide (SEQ ID NO: 3).

In additional exemplary embodiments, the compositions comprise the peptide N-acetyl-Cys-Met-Lys-Cys-amide (SEQ ID NO: 4).

In some embodiments, the peptide is in the form of a salt. In some embodiments, the salt is selected from the group consisting of trifluoroacetic acid (TFA), acetate and citrate salts. Each possibility represents a separate embodiment of the invention.

According to another aspect, the present invention provides at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1), and

Cys-Met-Lys-Cys (SEQ ID NO: 2);

for use in promoting mucus clearance.

According to yet another aspect, the present invention provides at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1), and

Cys-Met-Lys-Cys (SEQ ID NO: 2);

for use in the treatment of a disease or disorder where airway mucus is accumulated.

According to yet another aspect, the present invention provides the use of at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1), and

Cys-Met-Lys-Cys (SEQ ID NO: 2);

in the manufacture of a medicament for treating a disease or disorder where airway mucus is accumulated.

These and further aspects and features of the present invention will become apparent from the detailed description, examples and claims which follow.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the use of short peptides containing lysine and methionine located between two cysteine residues as mucolytic agents. The peptides are useful for promoting clearance of mucus that is accumulated in the respiratory tract and may interfere with breathing. The peptides are also useful for reducing symptoms of

diseases where airway mucus is accumulated, including for example, reducing of coughing.

Methods for reducing viscosity of mucus in airways of a subject to facilitate its clearance are provided. In addition, methods for treating diseases or disorders with excess mucus present in the respiratory tract are also provided.

Definitions

As used herein "peptide" indicates a sequence of amino acids linked by peptide bonds. In some embodiments, a peptide is composed of 10 amino acids or less, 9 amino acids or less, 8 amino acids or less, 7 amino acids or less, 6 amino acids or less, 5 amino acids or less. Each possibility represents a separate embodiment of the invention. In some embodiments, the peptide is composed of 4-10 amino acids, 4-9 amino acids, 4-8 amino acids, 4-7 amino acids, 4-6 amino acids, 4-5 amino acids, or 4 amino acids. Each possibility represents a separate embodiment of the invention. In some embodiments, a tetra-peptide is provided. The term "tetra-peptide" indicates a peptide composed of four amino acids. The peptides of the present invention are typically utilized in a linear form, although it will be appreciated that in cases where cyclization does not severely interfere with peptide characteristics, cyclic forms of the peptide can also be utilized.

The term “amino acid” refers to compounds, which have an amino group and a carboxylic acid group, preferably in a 1,2- 1,3-, or 1,4- substitution pattern on a carbon backbone. The term encompasses natural, non-natural and/or chemically modified amino acid residues. Natural amino acids include those found in proteins, which are L-amino acids. Non-natural and/or chemically modified amino acids include, for example, the corresponding N-methyl amino acids, side chain modified amino acids and the biosynthetically available amino acids which are not found in proteins (e.g., 5-hydroxy-lysine). The amino acid residues are represented throughout the specification and claims by either one or three-letter codes, as is commonly known in the art. The amino acids used in this invention are those which are available commercially or are available by routine synthetic methods. Certain residues may require special methods for incorporation into the peptide, and either sequential, divergent or convergent synthetic approaches to the peptide sequence are useful in this invention.

Also included within the scope of the invention are salts of the peptides, and derivatives of the peptides of the invention.

As used herein the term “salts” refers to salts of carboxyl groups and to acid addition salts of amino groups of the peptide molecule. Salts of carboxyl groups may be formed by means known in the art and include inorganic salts, for example sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases such as salts formed for example with amines such as triethanolamine, piperidine, procaine, and the like. Acid addition salts include, for example, salts with mineral acids such as, acetic acid or oxalic acid. Additional examples of suitable salts include trifluoroacetic acid (TFA), acetate and citrate salts.

Esters and amides of carboxy groups and acyl and alkyl derivatives of amino groups may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with terminal residues. Preferred chemical derivatives include peptides that have been C-termini amidated or N-termini acetylated.

"Derivatives” of the peptides of the invention as used herein covers derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e., they do not destroy the activity of the peptide, do not confer toxic properties on compositions containing it and do not adversely affect the antigenic properties thereof. These derivatives may, for example, include aliphatic esters of the carboxyl groups, amides of the carboxyl groups produced by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed by reaction with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups).

”Permeability“ refers to the ability of an agent or substance to penetrate, pervade, or diffuse through a barrier, membrane, or a skin layer. A “cell permeability”, “cell-penetration” or "permeability-enhancing" moiety refers to any molecule known in the art which is able to facilitate or enhance penetration of molecules through membranes. Non-limitative examples include: hydrophobic moieties such as lipids, fatty acids, steroids and bulky aromatic or aliphatic compounds. The permeability-enhancing moiety may be connected to any position in the peptide moiety, directly or through a spacer, preferably to the amino or carboxy terminus of the peptide moiety.

As used herein, "airway" or "airways" and "respiratory tract" refer to any part of the respiratory tract, including the nose, nasal cavity, pharynx, larynx, trachea, bronchi and lungs.

As used herein, "disease or disorder where airway mucus is accumulated" refers to medical conditions in which mucus accumulation in the respiratory tract is one of the symptoms. In such medical conditions mucus production may be excessive, thick, or otherwise difficult to clear from the respiratory tract.

As used herein "diseases or disorders where airway mucus is disturbing breathing" refers to medical conditions in which the presence of mucus in the airways, typically in excessive amounts, interferes with breathing and makes it difficult for the patient to breathe. Occasionally it may also cause coughing.

As used herein, "treating" and "treatment", refers to reduction, amelioration or even elimination of at least some of the symptoms associated with the relevant disease. For example, the term may include at least one of reducing sputum viscosity, improving cough and airway clearance of mucus, reducing accumulation of airway secretions, enabling better clearance by means of ciliary action, reducing airway obstruction, improving pulmonary function, improving gas exchange, reducing incidence of pulmonary exacerbations, facilitating expectoration, reducing or preventing repeated infection and airway damage. Each possibility represents a separate embodiment of the invention.

As used herein, the term "about", when referring to a measurable value such as an amount or size, is meant to encompass variations of +/-10%, more preferably 5 +/-5%, even more preferably +/-1%, and still more preferably +/-0.1% from the specified value, as such variations are appropriate to achieve the intended purpose.

Peptides

The present invention utilizes peptides and/or salts thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1), and

Cys-Met-Lys-Cys (SEQ ID NO: 2).

Each possibility represents a separate embodiment of the invention.

It is understood that Cys represents the amino-acid cysteine; Lys represents the amino-acid lysine; Met represents the amino-acid methionine.

The peptides utilized herein were disclosed in WO2012/098546, to the inventor of the present invention and others. Among other properties, their ability to reduce allergic responses in vivo was described. Their use as therapeutic agents for the treatment of allergic diseases, including allergic airways diseases such as asthma, was disclosed. Mucolytic activity of these peptides was not mentioned or suggested in WO2012/098546. It is now disclosed for the first time that these peptides can liquefy sputum samples effectively and rapidly, and can be used as mucolytic agents for promoting clearance of respiratory secretions and reducing symptoms of various diseases where respiratory secretions are difficult to clear.

In some embodiments, the peptide further comprises at least one modification selected from the group consisting of an amino-terminal modification and a carboxy-terminal modification. According to these embodiments, the peptide is selected from the group consisting of:

Z- Cys-Lys-Met-Cys- Y (SEQ ID NO: 5);

Z- Cys-Met-Lys-Cys- Y (SEQ ID NO: 6),

wherein Z is absent or represents an amino terminal modification and Y is absent or represents a carboxy terminal modification.

In some embodiments, the N- and C- termini modifications reduce the polarity of the peptides of the present invention, thus facilitating the ability of these peptides to cross cell membranes, enter easily into cells and accumulate within the cells. In addition, modifications of the peptide termini may improve bio-stability, for example by blocking the action of peptidases.

The amino and carboxy termini modifications may be chosen from any amino and carboxy termini modifications conventionally used in the art of peptide chemistry, which will not adversely affect the activities of the peptide.

In some embodiments, the amino terminal modification comprises addition of an amino terminal blocking group.

Blocking of the N terminus may be performed, for example, by alkylation or acylation, using methods well known in the art. Non-limiting examples of suitable N-terminal blocking groups include C_1-C_5 branched or unbranched alkyl groups, acyl groups such as formyl and acetyl groups, and substituted forms thereof, such as the acetamidomethyl (Acm) group. Each possibility represents a separate embodiment of the invention.

In some embodiments, the amino terminal modification comprises covalently linking to the N-terminus of the peptide a permeability-enhancing moiety that improves the ability of the peptide to penetrate lipid layers. In some exemplary embodiments, the moiety is a fatty acid. The fatty acid may be selected from the group consisting of palmitic acid, phosphatidic acid, stearic acid, arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid and oleic acid. Each possibility represents a separate embodiment of the invention.

In some typical embodiments, the amino terminal modification is selected from the group consisting of acetyl, alkyl, acyl and a fatty acid. Each possibility represents a separate embodiment of the invention.

In some embodiments, the carboxy terminal modification is a carboxy terminal blocking group.

Blocking of the C terminus may be performed, for example, by amidation, reduction or esterification, using methods well known in the art. Non-limiting examples of suitable C-terminal blocking groups include amide, ester, and alcohol groups. Each possibility represents a separate embodiment of the invention.

In some embodiments, the peptide is a tetra-peptide selected from the group consisting of: Cys-Lys-Met-Cys (SEQ ID NO: 1) and Cys-Met-Lys-Cys (SEQ ID NO: 2).

In some exemplary embodiments, the peptide N-acetyl-Cys-Lys-Met-Cys-amide (SEQ ID NO: 3) is provided.

In additional exemplary embodiments, the peptide N-acetyl-Cys-Met-Lys-Cys-amide (SEQ ID NO: 4) is provided.

The peptides may be synthesized by any technique known to those skilled in the art of peptide synthesis. These methods include solid phase as well as solution phase synthesis methods.

Solid phase peptide synthesis procedures are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

A skilled artesian may synthesize any of the peptides of the present invention by using an automated peptide synthesizer using standard chemistry such as, for example, t-Boc or Fmoc chemistry.

The methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis.

Synthetic peptides can be purified by preparative high performance liquid chromatography (Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.) and the composition of which can be confirmed via amino acid sequencing. Some of the peptides of the invention, which include only natural amino acids, may further be prepared using recombinant DNA techniques known in the art. The conjugation of the peptidic and permeability moieties may be performed using any methods known in the art, either by solid phase or solution phase chemistry. Some of the compounds of the present invention may conveniently be prepared using solution phase synthesis methods. Other methods known in the art to prepare compounds like those of the present invention can be used and are comprised in the scope of the present invention.

The permeability-enhancing moiety of the present invention may be connected to any position in the peptide moiety, directly or through a spacer. According to a specific embodiment, the cell-permeability moiety is connected to the amino terminus of the peptide moiety. The optional connective spacer may be of varied lengths and conformations comprising any suitable chemistry including but not limited to amine, amide, carbamate, thioether, oxyether, sulfonamide bond and the like. Non-limiting examples for such spacers include amino acids, sulfone amide derivatives, amino thiol derivatives and amino alcohol derivatives.

Cyclic versions of the peptides disclosed herein are also within the scope of the present invention. Cyclization of peptides may take place by any means known in the art, for example through free amino and carboxylic groups present in the peptide sequence, or through amino acids or moieties added for cyclization. Non limiting examples of cyclization types are: side chain to side chain cyclization (e.g., through S-S bonds), C-to-N terminal cyclization, side chain to terminal cyclization, and any type of backbone cyclization incorporating at least one N -ω-substituted amino acid residue/s as described for example in WO 95/33765.

Other methods known in the art to prepare peptides like those of the present invention can be used and are within the scope of the present invention.

In some embodiments, the peptide is in the form of a salt. Non-limiting examples of suitable salts include trifluoroacetic acid (TFA), acetate and citrate salts.

Compositions

The present invention provides compositions comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1), and

Cys-Met-Lys-Cys (SEQ ID NO: 2).

The compositions of the present invention may be formulated for local administration. For example, the compositions may be formulated for nasal administration e.g. as dropletts. As another example, they may formulated for inhalation administration. Formulations for inhalation may be provided, e.g., in the form of an aerosol spray from a pressurized metered dose inhaler with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide, in powder form administered using a dry powder inhaler or in aqueous liquid aerosol form using a nebulizer.

In some embodiments, a pharmacuetical composition suitable for inhalation administration is provided, comprising at least one peptide of the present invention or a salt thereof.

The compositions of the present invention may also be formulated for systemic administration. In some embodiments, they are formulated for oral administration. In other embodiments, they are fomulated for administration by injection.

Examples of administration routes include oral, rectal, intravenous, intramuscular, transdermal, subcutaneous, intradermal routes. Each possibility represents a separate embodiment of the invention.

The compositions and methods of the present invention are typically employed for the treatment of a mammal, preferably a human.

In some typical embodiments, the composition further comprises a pharmaceutically acceptable diluent, excipient or carrier.

As used herein, the term "pharmaceutically acceptable diluent, excipient, or carrier" refers to a diluent, excipient, or carrier that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered active agent.

As used herein, the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient.

Non-limiting examples of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, and polyethylene glycols. Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences", Mack Publishing Co., Easton, PA, (Remington: The Science and Practice of Pharmacy, Gennaro, A., Lippincott, Williams & Wilkins, Philadelphia, Pa., 20th ed, 2000).

In some embodiments, the therapeutic composition comprises a pharmaceutically acceptable carrier. As used herein, a "carrier" refers to any substance suitable as a vehicle for delivering of the agents or molecule of the present invention to a suitable in vivo or in vitro site. As such, carriers can act as a pharmaceutically acceptable excipient of a therapeutic composition of the present invention. Carriers of the present invention include: (1) excipients or formularies that transport, but do not specifically target a molecule to a cell (referred to herein as non-targeting carriers); and (2) excipients or formularies that deliver a molecule to a specific site in a subject or a specific cell (i.e., targeting carriers). Examples of non-targeting carriers include, but are not limited to water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters and glycols. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity.

Therapeutic compositions of the present invention can be sterilized by conventional methods.

In some embodiments, the composition further comprises at least one more active ingredient.

In some embodiments, a pharmaceutical composition is provided, consisting of the peptide of the present invention or a salt thereof as an active ingredient.

The peptide of the present invention or a salt thereof, and optionally additional one or more active ingredients, are present in the compositions of the present invention in an amount effective to achieve the intended purpose, for example, in an amount effective to treat a certain disease.

Pharmaceutical compositions of the present invention may be formulated in conventional manners. The proper formulation is dependent upon the route of administration chosen.

The compositions of the present invention may be formulated for sustained release of the active ingredient.

For administration by inhalation route, the active ingredients are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, for example, dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane, or carbon dioxide. In the case of a pressurized aerosol, the dosage may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base, such as lactose or starch.

For oral administration, enteric-coated preparations or dosage forms, microspheres, liposomes and nanoparticles for oral delivery of peptides and proteins may be used. Non-limiting examples of formulations for oral administration include tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Suitable carriers for oral administration are well known in the art. Compositions for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries as desired, to obtain tablets or dragee cores. Non-limiting examples of suitable excipients include fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol, cellulose preparations such as, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, and sodium carbomethylcellulose, and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate, may be added.

For administration by injection, the active ingredients of the composition may be formulated in aqueous solutions, for example in physiologically compatible buffers including but not limited to Hank's solution, Ringer's solution, or physiological salt buffer. Formulations for injection may be presented in unit dosage forms, for example, in ampoules, or in multi-dose containers with, optionally, an added preservative. The compositions may be in the form of suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing, and/or dispersing agents. Non-limiting examples of suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate,

triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the active ingredients, to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, for example, a sterile, pyrogen-free, water-based solution, before use.

The exact formulation, route of administration, and dosage can be chosen by the individual physician in view of the patient's condition.

In some embodiments, the composition further comprises at least one additive useful in the pharmaceutical fields, including, but not limited to fats, emulsifiers and co-emulsifiers, hydrophilic or lipophilic gelling agents, colorants, fragrances, emollients, humectants, preservatives, vitamins, chelators, solvents, fillers, thickeners, hydrophilic and lipophilic filters, dyestuffs, neutralizers, penetration-enhancing agents and polymers.

Non-limiting examples of suitable fats include mineral oils, oils of animal origin (lanolin), synthetic oils (isopropyl myristate, octyldodecyl, isostearyl isostearate, decyl oleate or isopropyl palmitate), silicone oils (cyclomethicone or dimethicone) and fluorinated oils. Fatty alcohol, fatty acids, waxes and gums, notably silicone gums and elastomers can also be used as fats.

Non-limiting examples of suitable emulsifiers and co-emulsifiers include polyglycerol fatty acid esters, sucrose fatty acid esters, sorbitane fatty acid esters, oxyethylene sorbitan fatty acid esters, PEG fatty alcohol ethers, glycerol fatty acid esters, alkyl sulphates, alkyl ether sulphates, alkyl phosphates, alkyl polyglucosides and dimethicone copolyols.

Non-limiting examples of suitable hydrophilic gelling include carboxyvinyl polymers (carbomer), acrylic copolymers such as acrylate/alkylacrylate copolymers, polyacrylamids, polysaccharides such as xanthan gum, guar gum, natural gums such as cellulose gum and derivatives, clays and 2-acrylamido-2-methylpropane acid copolymers.

Non-limiting examples of suitable lipophilic gelling agents include modified clays such as bentones, fatty acid metal salts, hydrophobic silica and ethylcellulose.

Non-limiting examples of suitable fillers include talc, kaolin, mica, serecite, magnesium carbonate, aluminum silicate and organic powders such as nylon.

Non-limiting examples of suitable dyestuffs include lipophilic dyes, hydrophilic dyes, pigments and mother-of-pearl commonly used in dermatological compositions, and their mixtures.

Non-limiting examples of suitable neutralizers include soda, triethanolamine, aminomethyl propanol and potassium hydroxide.

Non-limiting examples of suitable penetration enhancing agents include alcohols and glycols (ethanol and propylene glycol), ethoxydiglycol, alcohols and fatty acids (oleic acid), fatty acid esters and dimethyl isosorbide.

Non-limiting examples of preservatives compatible with pharmaceutical compositions include benzoic acid, its salts and esters, sorbic acid and its salts, parabens and their salts, triclosan, imidazolidinyl urea, phenoxyethanol, DMDM hydantoin, diazolidinyl urea and chlorphenesin.

Conventionally, the filters are UVA and UVB filters. Non-limiting examples of suitable UVA and UVB filters include organic filters such as benzophenone-3, butyl methoxydibenzoyl methane, octocrylene, octyl methoxycinnamate, 4-methylbenzylidene camphor, octyl salicylate, terephthalylidene dicamphor sulfonic acid and drometrizole trisiloxane, and non-organic filters such as titanium oxide and zinc oxide.

Non-limiting examples of suitable solvents include water, ethanol, glycerin, propylene glycol, butylene glycol and sorbitol.

The quantities and concentrations of these various additives are those conventionally used in pharmaceutical preparations as is known to a person skilled in the art.

Methods and uses

According to an aspect of the present invention, there is provided herein a method for assisting or improving mucus clearance from the respiratory tract of a subject in need thereof. In some embodiments, a method for reducing viscosity of airway mucus is provided. In some embodiments, the subject is afflicted with a disease or disorder where mucus accumulation is one of the symptoms or complications.

According to another aspect of the present invention, there is provided herein a method for treating a subject afflicted with a medical condition where airway mucus is accumulated. In some embodiments, a method for treating a subject afflicted with a

medical condition characterized by an imbalance between mucus secretion and clearance is provided.

The methods of the present invention comprise administering to a subject in need thereof a pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:

Cys-Lys-Met-Cys (SEQ ID NO: 1); and

Cys-Met-Lys-Cys (SEQ ID NO: 2).

In some embodiments, the disease or disorder is an infectious respiratory disease (including bacterial, fungal or viral).

In some embodiments, the disease or disorder is a degenerative lung disease. Degenerative lung diseases are typically characterized by damage to the lungs that progressively worsens over time and interferes with their ability to function.

In some embodiments, the disease or disorder is a genetic disease.

In some embodiments, the disease or disorder is an interstitial lung disease. Interstitial lung disease is a group of diseases that mainly affect the tissue and space around the alveoli (air sacs) (interstitium), most of which cause progressive scarring of lung tissue.

In some embodiments, the disease or disorder is acute sinusitis. In some embodiments, the disease or disorder is acute bronchitis. In some embodiments, the disease or disorder is bronchiolitis. In some embodiments, the disease or disorder is pneumonia. In some embodiments, the disease or disorder is tuberculosis. In some embodiments, the disease or disorder is COPD. In some embodiments, the disease or disorder is emphysema. In some embodiments, the disease or disorder is bronchiectasis. In some embodiments, the disease or disorder is chronic bronchitis. In some embodiments, the disease or disorder is CF. In some embodiments, the disease or disorder is primary ciliary dyskinesia. In some embodiments, the disease or disorder is secondary ciliary dyskinesia. In some embodiments, the disease or disorder is pulmonary fibrosis. In some embodiments, the disease or disorder is idiopathic pulmonary fibrosis. In some embodiments, the mucus accumulation is part of the pulmonary manifestations of systemic autoimmune diseases. In some embodiments, the disease or disorder is lung inflammation. In some embodiments, the disease or disorder is hyper-secretion of mucoproteins. In some embodiments, the excessive mucus is produced by the nasal mucosa. In some

embodiments, the disease or disorder is post-nasal drip. In some embodiments, the excessive mucus is produced in the lungs. In some embodiments, the mucus accumulation in the lungs is causing coughing. In some embodiments, the disease or disorder is pertussis.

In some embodiments, the method is used for assisting mucus clearance in a subject afflicted with an autoimmune disease, where the autoimmune disease is associated with airway mucus accumulation.

The methods of the present invention may also be utilized for the treatment of pulmonary complications associated with surgery, tracheostomy care, use during anesthesia, post-traumatic chest conditions and atelectasis due to mucous obstruction. Each possibility represents a separate embodiment of the invention.

In some embodiments, the methods of the present invention are used as an adjuvant therapy in respiratory conditions with excessive and/or thick mucus production.

Reduction of mucus viscosity may also be used for diagnostic bronchial studies (bronchograms, bronchospirometry, and bronchial wedge catheterization).

The methods of the present invention may be combined with one or more known treatments of the above described disorders/diseases.

In some embodiments, the methods of the present invention comprise administering a composition comprising at least one tetra-peptide of the present invention or a salt thereof.

According to certain embodiments, the methods comprise administering mixtures of peptides of the invention.

In some embodiments, the present invention provides the use of a peptide of the present invention or a salt thereof for the manufacture of a medicament for the treatment of a medical condition where airway mucus is accumulated.

In some embodiments, a pharmaceutical composition is provided, comprising a peptide of the present invention or a salt thereof as active ingredient, for use in the treatment of a medical condition where airway mucus is accumulated.

In some exemplary embodiments, the present invention provides the use of the peptide Cys-Lys-Met-Cys (SEQ ID NO: 1), in the treatment of a medical condition where airway mucus is accumulated.

In additional exemplary embodiments, the present invention provides the use of the peptide Cys-Met-Lys-Cys (SEQ ID NO: 2) in the treatment of a medical condition where airway mucus is accumulated.

The amount (dosage) of the pharmaceutical composition of the present invention to be administered for the above indications, the administration regimes as well as their mode of application will depend both on characteristics of the treated individual (age, size, gender, etc.) as well as on parameters associated with the phenomena to be treated.

The present invention further provides kits. In some embodiments, a kit is provided for promoting mucus clearance in a subject in need thereof. In some embodiments, a kit is provided, for treating a disease or disorder where airway mucus is accumulated. In some embodiments, a kit is provided, for reducing viscosity of airway mucus.

The aforementioned kits comprise a composition comprising at least one peptide of the present invention or a salt thereof, and may also include instructions for administering said composition to a subject in need thereof.

In some embodiments, the kit comprises means for administering the composition or compositions. For example, the kit may include a nebulizer, a face mask, an inhaler, a syringe, a dropper, a pipette or a combination thereof. Each possibility represents a separate embodiment of the invention.

In some embodiments, the kit comprises a composition comprising the at least one peptide or salt thereof (and optionally other active ingredients) dissolved in a suitable solvent. In other embodiments, the kit comprises a first composition comprising the peptide (and optionally other active ingredients), e.g. as a dried powder, and a second composition comprising a solvent.

The present invention can be further described by the following numbered clauses:

[1] A method for promoting mucus clearance in a subject in need thereof, the method comprising administering to the subject a mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of: Cys-Lys-Met-Cys (SEQ ID NO: 1); and Cys-Met-Lys-Cys (SEQ ID NO: 2).

[2] The method of clause 1, used for the treatment of a disease or disorder where airway mucus is accumulated or disturbing breathing.

[3] A method for treating a disease or disorder where airway mucus is accumulated or disturbing breathing, the method comprising administering to a subject in need thereof a mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of: Cys-Lys-Met-Cys (SEQ ID NO: 1); and Cys-Met-Lys-Cys (SEQ ID NO: 2).

[4] The method of any one of clauses 2-3, wherein the disease or disorder is an infectious respiratory disease.

[5] The method of clause 4, wherein the disease or disorder is selected form the group consisting of acute sinusitis, chronic sinusitis, acute bronchitis, bronchiolitis, pneumonia and tuberculosis.

[6] The method of any one of clauses 2-3, wherein the disease or disorder is a degenerative lung disease.

[7] The method of clause 6, wherein the disease or disorder is selected form the group consisting of chronic obstructive pulmonary disease (COPD), emphysema, bronchiectasis and chronic bronchitis.

[8] The method of any one of clauses 2-3, wherein the disease or disorder is a genetic disease.

[9] The method of clause 8, wherein the disease or disorder is ciliary dyskinesia.

[10] The method of clause 8, wherein the disease or disorder is cystic fibrosis (CF).

[11] The method of any one of clauses 2-3, wherein the disease or disorder is an interstitial lung disease.

[12] The method of clause 11, wherein the interstitial lung disease is a pulmonary fibrosis

[13] The method of any one of clauses 2-3, wherein the disease or disorder is lung inflammation.

[14] The method of any one of clauses 1-13, wherein the peptide further comprises at least one modification selected from the group consisting of an amino-terminal modification and a carboxy terminal modification.

[15] The method of clause 14, wherein the amino terminal modification is an amino terminal blocking group.

[16] The method of clause 15, wherein the amino-terminal blocking group is selected from the group consisting of alkyl and acyl.

[17] The method of clause 15, wherein the amino-terminal blocking group is an acetyl group.

[18] The method of clause 14, wherein the amino terminal modification is a moiety that improves the ability of the peptide to penetrate lipid layers.

[19] The method of clause 18, wherein the moiety is a fatty acid.

[20] The method of clause 19, wherein the fatty acid is selected from the group consisting of palmitic acid, phosphatidic acid, stearic acid, arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid and oleic acid.

[21] The method of clause 14, wherein the carboxy terminal modification is a carboxy terminal blocking group.

[22] The method of clause 21, wherein the carboxy terminal blocking group is selected from the group consisting of amide, ester and alcohol group.

[23] The method of clause 22, wherein the carboxy terminal blocking group is an amide group.

[24] The method of any of clauses 1-23, wherein the peptide is in the form of a salt.

[25] The method of the preceding clauses, wherein the peptide is a tetra-peptide.

[26] A mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of: Cys-Lys-Met-Cys (SEQ ID NO: 1); and Cys-Met-Lys-Cys (SEQ ID NO: 2) for use in promoting mucus clearance.

[27] A mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of: Cys-Lys-Met-Cys (SEQ ID NO: 1); and Cys-Met-Lys-Cys (SEQ ID NO: 2) for use in the treatment of a disease or disorder where airway mucus is accumulated or disturbing breathing.

[28] The pharmaceutical composition of any one of clauses 26-27, formulated for systemic administration.

[29] The pharmaceutical composition of any one of clauses 26-27, formulated for local administration.

[30] The pharmaceutical composition of clause 29, formulated for inhalation or nasal administration.

The following examples are presented in order to more fully illustrate certain embodiments of the invention. They should in no way, however, be construed as limiting the broad scope of the invention. One skilled in the art can readily devise many variations and modifications of the principles disclosed herein without departing from the scope of the invention.

EXAMPLES

Example 1

The following peptides were synthesized by Solid Phase Peptide Synthesis (SPSS) using Fmoc strategy (purity >98%), as disclosed in WO 2012/098546:

N-acetyl-Cys-Lys-Met-Cys-NH_2, designated herein as DY-65 (SEQ ID NO: 3);

N-acetyl-Cys-Met-Lys-Cys-NH_2, designated herein as DY-70 (SEQ ID NO: 4).

N-acetyl-Cys-βAla-His-Cys-NH_2, designated herein as DY-66 (SEQ ID NO: 7).

The peptides were prepared by SPSS in which there are repeated cycles of coupling-deprotection. The first stage of the technique consists of peptide chain assembly with protected amino acid derivatives on a polymeric support. The second stage of the technique is the cleavage of the peptide from the resin support with the concurrent cleavage of all side chain protecting groups to give the crude free peptide.

The free N-terminal amine of a solid-phase attached peptide is first coupled to a single N-protected amino acid unit. This unit is then deprotected, revealing a new N-terminal amine to which a further amino acid was attached. After cleavage from the resin, peptides are then purified by reverse phase HPLC using columns.

Fmoc deprotection: 0.08 mmol of Fmoc-X-Wang resin is loaded into a fritted column equipped with a plastic cap. The resin is washed twice with 3 mL portions of dimethylformamide (DMF) for 1 minute each. Next, 3 ml of 20% piperidine in DMF is added and deprotection allowed to continue for 15 minutes. During this time, the column is gently swirled in order to assure a complete mixing. After the reaction is complete (in about 15 minutes), the reaction column is drained and the resin washed 4 times with 3 mL of DMF.

Amide bond coupling: In a small vial, 3 equivalents of the Fmoc amino acid is preactivated by combining it with equal equivalents of O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 6 equivalents of DIPEA (N,N'-diisopropylethylamine), and 3 mL of DMF. This solution is fully dissolved

and then allowed to react for an additional 3-5 minutes. Then this coupling solution is added to the resin. The cap is placed on the reaction column and the resin slurry agitated every 2-3 minutes over a period of 20 minutes.

Cleavage: In order to obtain the peptide in the free acid form, the ester linkage is cleaved using trifluoroacetic acid (TFA). The resin is treated with 2-3 mL of a solution of TFA and water in a ratio of 95:5. The resin is then agitated over a period of 25 minutes. The column is subsequently drained and the filtrated collected into a glass collection vessel. The material is then dried in diethyl ether and analyzed.

Testing mucolytic activity

The above peptides were tested for mucolytic activity on sputum (mucus) samples ex-vivo in comparison to N-acetylcysteine (NAC) and dithiothreitol (DTT).

Induced sputum is a well established non invasive procedure to retrieve inflammatory specimens from the lungs. The specimens contain, *inter alia*, leukocytes and other cells in an aggregate of mucins, antiseptic enzymes, immunoglobulins, lactoferrin and additional macromolecules. In certain diseases the sputum samples may contain also substantial amount of DNA and/or Bacteria. Upon treatment of the specimens with a mucolytic agent, the bonds between the mucu-proteins and also between other different components are disrupted, the cellular material is released (separated from the sputum) and the mucus liquefies. The cellular material can be collected and various types of cells can be counted, to yield a profile of cell distribution in the specimen (i.e., absolute number and percentage of each type of cell within the total cell count).

Viscid selected portions of sputum specimens (plugs) that are treated with DTT typically produce final samples that contain well defined cellular material with no contamination of viscous debris. NAC produces a similar mucolytic effect in vitro on sputum samples.

In the present study, the differential cell distribution obtained after treatment of sputum samples with each of the peptides DY-65, DY-70 and DY-66 described above was compared to that obtained with DTT and NAC. Each peptide was tested under various conditions, as detailed below. Liquefaction of sputum samples, along with differential cell distribution similar to that of DTT and NAC, is indicative of mucolytic activity of the peptides.

Sputum samples were collected from subjects with airway mucus accumulation. Sputum was processed as soon as possible within 2 hours of collection. The samples were split into equal aliquots and treated with Sputolysin®, NAC and each of the three tested peptides, as follows:

Sputolysin® (DTT concentrate in phosphate buffer, pH 6.5-7.5, Calbiochem Corp., San Diego, CA, USA): Sputolysin® prepared in a dilution of 1:10 s with distilled water according to the manufacturer's instructions was added and mixed mechanically with the sputum in a shaking water bath at 37°C for complete homogenization. Following incubation, a double volume of PBS was added to stop the reaction, and the cell suspension containing the phosphate-buffered solution was filtered through a 52 μm nylon gauze (BNSH Thompson, Scarborough, Ontario, Canada) and diluted with RPMI supplemented with fetal calf serum (FCS) to achieve a concentration of 10^3 cells/μl. Next, two drops were placed in a cytocentrifuge cup already in place in a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA), and cytospins were prepared at 1000 RPMI supplemented with 10% FCS (Biological Industries, Beit Haemek, Israel) for 5 minutes. Separate cytospin slides were stained by Giemsa. Four hundred (400) nonsquamous cells were counted. The number of neutrophils, eosinophils, mast cells, lymphocytes and macrophages was determined, and the results were expressed as a percentage of the total nonsquamous cell count. Only samples containing ≤20% squamous cells were used.

NAC: NAC ($HSCH_2CH(NHCOCH_3)CO_2H$ Sigma Grade >99% (TLC) powder Cat No A9165-5G (cell culture tested)) was prepared as follows: 10% NAC were mixed with 1 ml NaOH 0.01M, 1ml 0.025M Tri Sodium Citrate, adjusted to pH~7. Sputum and NAC at 1:1 or 1:2 volume ratio were mixed mechanically in a shaking water bath at 37°C for the time periods indicated in the tables below, for complete homogenization. Further processing and cell counts were performed as described above for Sputolysin®.

Peptides: DY-65 and DY-70 were freshly prepared from aliquots of 5mg/ml at a final concentration of 1% or 2%, in water. In some experiments they were prepared in saline or PBS. DY-66 was prepared from aliquots of 5mg/ml in three different concentrations 1%, 5% and 25% in distilled water together with NaOH and Trisodium bicarbonate to arrive to normalization of pH. Sputum and peptide at 1:1 or 1:2 volume ratio were mixed mechanically in a shaking water bath at 37°C for the time periods

indicated in the tables below. Further processing and cell counts were performed as described above.

The results are summarized in Tables 1-7 below.

Results:

DY-65 and DY-70

Table 1- DY-65 and DY-70 versus Sputolysin®

	Compound DY-65 (1%)		**Compound DY-70 (1%)**	
n=400cells counted (%)	**5' incubation dilution of mixture 1:1* pH 4.9, in water**	**Sputolysin®**	**5' incubation dilution of mixture 1:1* pH 2.4, in water**	**Sputolysin®**
Neutrophils	303 (75.5%)	270 (67.5%)	296 (74%)	305 (76.25%)
Eosinophils	22 (5.5%)	29 (7.2%)	12 (3.0%)	17 (4.25%)
Mast Cells	1 (0.25%)	1 (0.25%)	1 (0.25%)	-
Lymphocytes	30 (7.5%)	34 (8.5%)	63 (15.75%)	48 (12%)
Macrophages	44 (11%)	66 (16.5%)	28 (7.0%)	30 (7.5%)

* 1:1 means equal volumes between sputum and compound DY-65 or DY-70.

Table 2- DY-65 and DY-70 versus NAC- Experiment 1

n =400 cells counted (%)	**NAC 10' incubation dilution of mixture 1:2 * pH 6.75**	**NAC 10' incubation dilution of mixture 1:2 * pH 7.37**	**Compound DY-65 (1%) 10' incubation dilution of mixture 1:2 * pH 4.9, in water**	**Compound DY-70 (1%) 10' incubation Dilution of mixture 1:1* pH 2.4, in water**
Neutrophils	303 (75.5%)	299 (74.75%)	252 (63%)	250 (62.5%)
Eosinophils	22 (5.5%)	21 (5.25%)	32 (8.0%)	21 (5.25%)
Mast Cells	1 (0.25%)	-	1 (0.25%)	-
Lymphocytes	30 (7.5%)	60 (15%)	64 (16%)	70 (17.5%)
Macrophages	44 (11%)	30 (7.5%)	51 (12.75%)	59 (14.75%)

* 1:2 means one volume sputum in two volumes compound DY-65/DY-70 or NAC

Table 3- DY-65 and DY-70 versus NAC – Experiment 2

n =400 cells counted (%)	**NAC 10' incubation dilution of mixture 1:2 * pH 6.98**	**Compound DY-65 (1%) 10' incubation dilution of mixture 1:2* pH 4.9, in water**	**Compound DY-70 (1%) 10' incubation Dilution of mixture 1:1* pH 2.4, in water**
Neutrophils	238 (59.5%)	182 (45.5%)	192 (48%)
Eosinophils	1 (0.25%)	2 (0.5%)	2 (0.5%)
Mast Cells	-	-	-
Lymphocytes	51 (12.75%)	69 (17.25%)	56 (14%)
Macrophages	110 (27.5%)	147 (36.75%)	150 (37.5%)

* 1:2 means one volume sputum in two volumes compound DY-65/DY-70 or NAC

Table 4- DY-65 and DY-70 versus NAC– Experiment 3

n =400 cells counted (%)	**NAC 10' incubation dilution of mixture 1:2 * pH 6.98**	**Compound DY-65 (1%) 10' incubation dilution of mixture 1:2* pH 4.9, in water**	**Compound DY-70 (1%) 10' incubation Dilution of mixture 1:1* pH 2.4, in water**
Neutrophils	301 (75.25%)	280 (70%)	305 (76.25%)
Eosinophils	2 (0.5%)	2 (0.5%)	1 (0.25%)
Mast Cells	-	-	-
Lymphocytes	56 (14%)	58 (14.5%)	53 (13.25%)
Macrophages	41 (10.25%)	60 (15%)	41 (10.25%)

* 1:2 means one volume sputum in two volumes compound DY-65/DY-70 or NAC

Table 5 – DY-65 in saline or PBS versus NAC

	Compound DY-65 (1%)		**NAC**
n=400 cells counted (%)	**20' incubation dilution of mixture 1:1* in saline pH 6.05**	**20' incubation dilution of mixture 1:1** in PBS pH 6.67**	**20' incubation dilution of mixture 1:1* pH 6.07**
Neutrophils	322 (80.5%)	347 (86.75%)	347 (86.75%)
Eosinophils	6 (1.5%)	7 (1.75%)	6 (1.5%)
Mast Cells	1 (0.25%)	1 (0.25%)	-
Lymphocytes	48 (12.0%)	29 (7.25%)	27 (6.75%)
Macrophages	23 (5.75%)	16 (4.0%)	20 (5.0%)

* 1:1 means equal volumes between sputum and compound DY-65 or NAC

Table 6 – DY-70 (1%) in PBS

n =400 cells counted (%)	**7' incubation dilution of mixture 1:2 * in PBS pH 6.5**	**14' incubation dilution of mixture 1:2 * in PBS pH 6.5**
Neutrophils	356 (89.0%)	321 (80.25%)
Eosinophils	1 (0.25%)	2 (0.25%)
Mast Cells	-	-
Lymphocytes	34 (8.5%)	44 (11.0%)
Macrophages	29 (7.25%)	33 (8.25%)

* 1:2 means one volume sputum in two volumes compound DY-70

Table 7 - DY-65 in different concentrations in PBS versus NAC

n =400 cells counted (%)	**NAC 10' incubation dilution of mixture 1:2* pH 7.16**	**Compound DY-65 (1%) 10' incubation dilution of mixture 1:2* pH 6.5**	**Compound DY-65 (2%) 10' incubation dilution of mixture 1:2* pH 5.9**
Neutrophils	383 (95.7)	388 (97)	382 (95.5)
Eosinophils	-	-	-
Mast Cells	-	-	-
Lymphocytes	9 (2.25)	8 (2)	8 (2)
Macrophages	8 (2)	4 (1)	10 (2.5)

* 1:2 means one volume sputum in two volumes compound DY-65 or NAC

Conclusions: The compounds DY-65 and DY-70 have a good mucolytic effect on sputum specimens and render a similar differential cell distribution as Sputolysin® and NAC preparations.

These two peptides showed complete liquefaction of the sputum samples, within a shorter period of time compared to NAC.

DY-66

Preparations of DY-66, prepared as described above in three different concentrations 1%, 5% and 25%, were each added to 0.5 gr sputum and mixed mechanically in a shaking water bath at 37°C for 15 minutes. Sputum was hardly homogenized. The cells were counted but morphology was not optimally preserved. Thus, no mucolytic activity was observed for DY-66 under the examined conditions.

Example 2 – Mucolytic effect on sputum samples from cystic fibrosis patients

Peptides DY-65 and DY-70 are tested in-vitro for mucolytic activity on sputum samples taken from cystic fibrosis (CF) patients. Sputum from CF patients is particularly thick and viscous and contains, *inter alia*, DNA, F-actin and other products of inflammation.

Sputum samples are collected from subjects with CF. Sputum is processed as soon as possible within 2 hours of collection. The samples are split into equal aliquots and treated with DY-65 or DY-70 preparations containing the peptide at a concentration ranging from 1-10% w/v, for example, 1%, 2%, 5% and 10% w/v, in water, saline or PBS.

Sputum and peptide at 1:1 or 1:2 volume ratio are mixed mechanically in a shaking water bath at 37°C for time periods ranging from 5-25 minutes, for example 5 minutes, 10 minutes, 15 minutes, 20 minutes and 25 minutes.

Further processing and cell counts are performed as described above in Example 1. The number of neutrophils, eosinophils, mast cells, lymphocytes and macrophages is determined, and the results are expressed as a percentage of the total nonsquamous cell count. Only samples containing ≤20% squamous cells are used.

The activity of the peptides is compared to that of N-acetylcysteine (NAC) and/or dithiothreitol (DTT) prepared as described above.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not

of limitation. The means, materials, and steps for carrying out various disclosed chemical structures and functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. A method for promoting mucus clearance from a respiratory tract of a subject in need thereof, the method comprising administering to the subject a mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:
   Cys-Lys-Met-Cys (SEQ ID NO: 1); and
   Cys-Met-Lys-Cys (SEQ ID NO: 2).

2. The method of claim 1, used for the treatment of a disease or disorder where airway mucus is accumulated or disturbing breathing.

3. A method for treating a disease or disorder where airway mucus is accumulated or disturbing breathing, the method comprising administering to a subject in need thereof a mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:
   Cys-Lys-Met-Cys (SEQ ID NO: 1); and
   Cys-Met-Lys-Cys (SEQ ID NO: 2).

4. The method of any one of claims 2-3, wherein the disease or disorder is an infectious respiratory disease.

5. The method of claim 4, wherein the disease or disorder is selected form the group consisting of acute sinusitis, chronic sinusitis, acute bronchitis, bronchiolitis, pneumonia and tuberculosis.

6. The method of any one of claims 2-3, wherein the disease or disorder is a degenerative lung disease.

7. The method of claim 6, wherein the disease or disorder is selected form the group consisting of chronic obstructive pulmonary disease (COPD), emphysema, bronchiectasis and chronic bronchitis.

8. The method of any one of claims 2-3, wherein the disease or disorder is a genetic disease.

9. The method of claim 8, wherein the disease or disorder is ciliary dyskinesia.

10. The method of claim 8, wherein the disease or disorder is cystic fibrosis (CF).

11. The method of any one of claims 2-3, wherein the disease or disorder is an interstitial lung disease.

12. The method of claim 11, wherein the interstitial lung disease is a pulmonary fibrosis

13. The method of any one of claims 2-3, wherein the disease or disorder is lung inflammation.

14. The method of any one of claims 1-13, wherein the peptide further comprises at least one modification selected from the group consisting of an amino-terminal modification and a carboxy terminal modification.

15. The method of claim 14, wherein the amino terminal modification is an amino terminal blocking group.

16. The method of claim 15, wherein the amino-terminal blocking group is selected from the group consisting of alkyl and acyl.

17. The method of claim 15, wherein the amino-terminal blocking group is an acetyl group.

18. The method of claim 14, wherein the amino terminal modification is a permeability-enhancing moiety that improves the ability of the peptide to penetrate lipid layers.

19. The method of claim 18, wherein the permeability-enhancing moiety is a fatty acid.

20. The method of claim 19, wherein the fatty acid is selected from the group consisting of palmitic acid, phosphatidic acid, stearic acid, arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid and oleic acid.

21. The method of claim 14, wherein the carboxy terminal modification is a carboxy terminal blocking group.

22. The method of claim 21, wherein the carboxy terminal blocking group is selected from the group consisting of amide, ester and alcohol group.

23. The method of claim 22, wherein the carboxy terminal blocking group is an amide group.

24. The method of any of claims 1-23, wherein the peptide is in the form of a salt.

25. The method of any one of claim 1-23, wherein the peptide is of 4-10 amino acids.

26. The method of any of claims 1-23, wherein the peptide is a tetra-peptide.

27. A mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:
Cys-Lys-Met-Cys (SEQ ID NO: 1); and
Cys-Met-Lys-Cys (SEQ ID NO: 2),
for use in promoting mucus clearance.

28. A mucolytic pharmaceutical composition comprising as an active ingredient at least one peptide or a salt thereof having an amino acid sequence selected from the group consisting of:
Cys-Lys-Met-Cys (SEQ ID NO: 1); and
Cys-Met-Lys-Cys (SEQ ID NO: 2)
for use in the treatment of a disease or disorder where airway mucus is accumulated or disturbing breathing.

29. The pharmaceutical composition of any one of claims 27-28, formulated for systemic administration.

30. The pharmaceutical composition of any one of claims 27-28, formulated for local administration.

31. The pharmaceutical composition of claim 30, formulated for inhalation or nasal administration.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2015/050144

A. CLASSIFICATION OF SUBJECT MATTER

IPC (2015.01) A61K 38/07, C07K 5/10, A61P 11/00, A61P 11/12

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)

IPC (2015.01) A61K, C07K, A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases consulted: BLAST, THOMSON INNOVATION, Google Patents, CAPLUS, MEDLINE, REGISTRY, Google Scholar
Search terms used: sequence searc, Cxxc motif, mucus, mucolytics, tetrapeptide, peptide, Thioreduxine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004024868 A2 NAT JEWISH MED & RES CENTER?[US]; WHITE CARL W [US] 25 Mar 2004 (2004/03/25) page 9 second and last paragraphs, page 16 second paragraph	1-13,25-31
Y	page 9 second and last paragraphs, page 16 second paragraph	14-24
Y	WO 2012098546 A2 ONEDAY BIOTECH AND PHARMA LTD?[IL]; YISSUM RES DEV CO?[IL]; MOGRABI JOSEJ?[IL]; ATLAS DAPHNE?[IL]; KEYNAN SHOSHANA?[IL] 26 Jul 2012 (2012/07/26) cited in the document	14-24

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* 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 application or patent but 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	Date of mailing of the international search report
14 May 2015	17 May 2015
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616	Authorized officer RON-COHEN Yael Telephone No. 972-2-5651737

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IL2015/050144

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IL2015/050144

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
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