(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 July 2006 (20.07.2006)

PCT

(10) International Publication Number
WO 2006/076254 A2

(51) International Patent Classification:
A61K 38/08 (2006.01)

(21) International Application Number:
PCT/US2006/000592

(22) International Filing Date: 10 January 2006 (10.01.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/642,520 11 January 2005 (11.01.2005) US

(71) Applicant *(for all designated States except US)*: **REGENERX BIOPHARMACEUTICALS, INC.** [US/US]; 3 Bethesda Metro Center, Suite 630, Bethesda, Maryland 20814 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GOLDSTEIN, Allan, L.** [US/US]; 800 25th Street, N.W., Apt. 1005, Washington, District Of Columbia 20037 (US). **FINKELSTEIN, Jack, Jr.** [US/US]; 3703 Taylor Street, Chevy Chase, Maryland 20815 (US). **CROCKFORD, David** [US/US]; 62 Kent Street, Newburyport, Massachusetts 01950 (US).

(74) Agents: **REPPER, George, R.** et al.; Rothwell, Figg, Ernst & Manbeck, 1425 K Street, NW, Suite 800, Washington, District Of Columbia 20005 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/076254 A2

(54) Title: METHOD OF TREATING OR PREVENTING RESPIRATORY MICROBIAL INFECTION OF RESPIRATORY TISSUE

(57) Abstract: A method of treatment for treating, preventing, inhibiting or reducing respiratory microbial infection of respiratory tissue of a subject, includes administering to a subject in need of such treatment an effective amount of a composition including an antimicrobial agent including amino acid sequence LKKTET or LKKTNT, a conservative variant thereof, or an agent that stimulates production of an LKKTET or LKKTNT peptide, or a conservative variant thereof, in the tissue, so as to inhibit the infection.

METHOD OF TREATING OR PREVENTING RESPIRATORY MICROBIAL INFECTION OF RESPIRATORY TISSUE

BACKGROUND OF THE INVENTION

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 60/642,520, filed January 11, 2005.

Field of the Invention

[0002] The present invention relates to the field of treating or preventing respiratory microbial infection of respiratory tissue.

Description of the Background Art

[0003] Respiratory microbial infections, particularly respiratory bacterial infections, can be dangerous and even life-threatening.

[0004] Respiratory infections caused by *Pseudomonas aeruginosa* and other gram-negative bacteria occur almost exclusively in individuals with a compromised lower respiratory tract or a compromised systemic defense mechanism. Primary pneumonia occurs in patients with chronic lung disease and congestive heart failure. Bacteremic pneumonia commonly occurs in neutropenic cancer patients undergoing chemotherapy. Lower respiratory tract colonization of cystic fibrosis patients by mucoid strains of *Pseudomonas aeruginosa* and other gram-negative bacteria is common and difficult, if not impossible, to treat.

[0005] There remains a need in the art for methods of treatment for treating, preventing, inhibiting or reducing respiratory microbial infections.

SUMMARY OF THE INVENTION

[0006] In accordance with one aspect, a method of treatment for treating, preventing, inhibiting or reducing respiratory microbial infection of respiratory tissue of a subject, comprises administering to a subject in need of such treatment an effective amount of a composition comprising an antimicrobial agent comprising amino acid sequence LKKTET or LKKTNT, a conservative variant thereof, or a stimulating agent that stimulates production of an LKKTET or LKKTNT peptide, or a conservative variant thereof, in said tissue, so as to inhibit said microbial infection.

DETAILED DESCRIPTION OF THE INVENTION

[0007] Without being found to any specific theory, actin-sequestering peptides such as thymosin beta 4 (Tβ4 or TB4) and other agents including actin-sequestering peptides or peptide fragments containing amino acid sequence LKKTET or LKKTNT or conservative variants thereof, promote reversal or prevention of respiratory infection of respiratory tissue.

[0008] In preferred embodiments, a respiratory infection treated in accordance with the present invention is a bacterial infection, more preferably a gram-negative bacterial infection, and most preferably a *Pseudomonas aeruginosa* respiratory infection.

[0009] A subject being treated in accordance with the present invention preferably is mammalian, most preferably human.

[0010] Thymosin 4 was initially identified as a protein that is up-regulated during endothelial cell migration and differentiation *in vitro*. Thymosin 4 was originally isolated from the thymus and is a 43 amino acid, 4.9 kDa ubiquitous polypeptide identified in a variety of tissues. Several roles have been ascribed to this protein including a role in a endothelial cell differentiation and migration, T cell differentiation, actin sequestration, vascularization and wound healing.

[0011] In accordance with one embodiment, the invention is a method of treatment for treating, preventing, inhibiting or reducing respiratory microbial infection of respiratory tissue of a subject, comprising administering to a subject in need of such treatment an effective amount of a composition comprising an antimicrobial agent, which may be a polypeptide comprising amino acid sequence LKKTET or LKKTNT, or a conservative variant thereof having antimicrobial activity, preferably Thymosin β4, and/or Tβ4 isoforms, analogues or derivatives, including KLKKTET, LKKTETQ, N-terminal variants of Tβ4, C-terminal variants of Tβ4 and antagonists of Tβ4. The invention also may utilize oxidized Tβ4. In accordance with other embodiments, the antimicrobial agent is other than thymosin beta 4 or oxidized Tβ4.

[0012] Compositions which may be used in accordance with the present invention include agents such as Thymosin β4 (Tβ4), and/or Tβ4 isoforms, analogues or derivatives, oxidized Tβ4, N-terminal variants of Tβ4, C-terminal variants of Tβ4 and antagonists of Tβ4, polypeptides or peptide fragments comprising or consisting essentially of the amino acid sequence LKKTET or conservative variants thereof, having antimicrobial activity. International Application Serial No. PCT/US99/17282, incorporated herein by reference, discloses isoforms of Tβ4 which may be useful in

accordance with the present invention as well as amino acid sequence LKKTET and conservative variants thereof, which may be utilized with the present invention. International Application Serial No. PCT/GB99/00833 (WO 99/49883), incorporated herein by reference, discloses oxidized Thymosin β4 which may be utilized in accordance with the present invention. Although the present invention is described primarily hereinafter with respect to Tβ4 and Tβ4 isoforms, it is to be understood that the following description is intended to be equally applicable to amino acid sequence LKKTET or LKKTNT, peptides and fragments comprising or consisting essentially of LKKTET or LKKTNT, conservative variants thereof having antimicrobial activity, and/or Tβ4 isoforms, analogues or derivatives, including N-terminal variants of Tβ4, C-terminal variants of Tβ4 and antagonists of Tβ4. The invention also may utilize oxidized Tβ4. The antimicrobial agent may be directly or indirectly antimicrobial.

[0013] In one embodiment, the invention provides a method of treatment for treating, preventing, inhibiting or reducing respiratory microbial infection of respiratory tissue of a subject, by contacting the tissue with an antimicrobial effective amount of a composition which contains an antimicrobial agent as described herein. As non-limiting examples, the tissue may be selected from respiratory tract or airway tissue of said subject. The contacting may be directly or systemically. Examples of direct administration include, for example, contacting the tissue, by direct application or inhalation, with a solution, lotion, salve, gel, cream, paste, spray, suspension, dispersion, hydrogel, ointment, or oil comprising an antimicrobial agent as described herein. Systemic administration includes, for example, intravenous, intraperitoneal, intramuscular injections or infusions of a composition containing an antimicrobial agent as described herein, in a pharmaceutically acceptable carrier such as water for injection.

[0014] Antimicrobial agents for use in the invention, as described herein, may be administered in any suitable microbial infection-inhibiting amount. For example, an antimicrobial agent as described herein may be administered in dosages within the range of about 0.0001-1,000,000 micrograms, more preferably in amounts within the range of about 0.1-5,000 micrograms, most preferably within the range of about 1-30 micrograms.

[0015] A composition in accordance with the present invention can be administered daily, every other day, every other week, every other month, etc., with a single application or multiple applications per day of administration, such as applications 2, 3, 4 or more times per day of administration.

[0016] Many Tβ4 isoforms have been identified and have about 70%, or about 75%, or about 80% or more homology to the known amino acid sequence of Tβ4. Such isoforms include, for example, $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15. Similar to Tβ4, the Tβ10 and Tβ15 isoforms have been shown to sequester actin. Tβ4, Tβ10 and Tβ15, as well as these other isoforms share an amino acid sequence, LKKTET or LKKTNT, that appears to be involved in mediating actin sequestration or binding. For example, Tβ4 can modulate actin polymerization (e.g. β-thymosins appear to depolymerize F-actin by sequestering free G-actin). Tβ4's ability to modulate actin polymerization may therefore be due to all, or in part, its ability to bind to or sequester actin via the LKKTET sequence. Thus, as with Tβ4, other proteins which bind or sequester actin, or modulate actin polymerization, including Tβ4 isoforms having the amino acid sequence LKKTET, are likely to be effective, alone or in a combination with Tβ4, as set forth herein.

[0017] Thus, it is specifically contemplated that known Tβ4 isoforms, such as $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15, as well as Tβ4 isoforms not yet identified, will be useful in the methods of the invention. As such Tβ4 isoforms are useful in the methods of the invention, including the methods practiced in a subject. The invention therefore further provides pharmaceutical compositions comprising Tβ4, as well as Tβ4 isoforms $T\beta4^{ala}$, Tβ9, Tβ10, Tβ11, Tβ12, Tβ13, Tβ14 and Tβ15, and a pharmaceutically acceptable carrier.

[0018] In addition, other antimicrobial agents or proteins having actin sequestering or binding capability, or that can mobilize actin or modulate actin polymerization, as demonstrated in an appropriate sequestering, binding, mobilization or polymerization assay, or identified by the presence of an amino acid sequence that mediates actin binding, such as LKKTET or LKKTNT, for example, can similarly be employed in the methods of the invention. Such proteins may include gelsolin, vitamin D binding protein (DBP), profilin, cofilin, depactin, DnaseI, vilin, fragmin, severin, capping protein, β-actinin and acumentin, for example. As such methods include those practiced in a subject, the invention further provides pharmaceutical compositions comprising gelsolin, vitamin D binding protein (DBP), profilin, cofilin, depactin, DnaseI, vilin, fragmin, severin, capping protein, β-actinin and acumentin as set forth herein. Thus, the invention includes the use of an antimicrobial polypeptide comprising the amino acid sequence LKKTET or LKKTNT and conservative variants thereof.

[0019] As used herein, the term "conservative variant" or grammatical variations thereof denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the replacement of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, the replacement of a polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

[0020] Tβ4 has been localized to a number of tissue and cell types and thus, agents which stimulate the production of an LKKTET or LKKTNT peptide such as Tβ4 or another antimicrobial agent as described herein, can be added to or comprise a composition to effect production an antimicrobial agent from a tissue and/or a cell. Such stimulating agents may include members of the family of growth factors, such as insulin-like growth factor (IGF-1), platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), basic fibroblast growth factor (bFGF), thymosin α1 (Tα1) and vascular endothelial growth factor (VEGF). More preferably, the stimulating agent is transforming growth factor beta (TGF-β) or other members of the TGF-β superfamily.

[0021] In accordance with one embodiment, subjects are treated with a stimulating agent that stimulates production in the subject of an antimicrobial agent as defined herein.

[0022] Additionally, other agents that assist in reduction of respiratory microbial infection of respiratory tissue may be added to a composition along with an agent as described herein. For example, and not by way of limitation, an antimicrobial agent as described herein alone or in combination can be added in combination with any one or more of the following agents: antibiotics, VEGF, KGF, FGF, PDGF, TGFβ, IGF-1, IGF-2, IL-1, prothymosin α and/or thymosin α1 in an effective amount.

[0023] The invention also includes a pharmaceutical composition comprising a therapeutically effective amount of an antimicrobial agent as described herein in a pharmaceutically acceptable carrier such as water for injection.

[0024] The actual dosage or reagent, formulation or composition that provides treatment may depend on many factors, including the size and health of a subject. However, persons of ordinary skill in the art can use teachings describing the methods and techniques for determining clinical dosages as disclosed in PCT/US99/17282, *supra*, and the references cited therein, to determine the appropriate dosage to use.

[0025] Suitable formulations may include an antimicrobial agent as described herein at a concentration within the range of about 0.001 - 50% by weight, more preferably

within the range of about 0.01 - 0.1% by weight, most preferably about 0.05% by weight.

[0026] The therapeutic approaches described herein involve various routes of administration or delivery of an antimicrobial agent as described herein, including any conventional administration techniques (for example, but not limited to, direct administration, local injection, inhalation, or systemic administration), to a subject. The methods and compositions using or containing an antimicrobial agent as described herein may be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients or carriers.

[0027] The invention may include use of antibodies which interact with an antimicrobial agent as described herein. Antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known to those skilled in the art as disclosed in PCT/US99/17282, *supra*. The term antibody as used in this invention is meant to include monoclonal and polyclonal antibodies.

[0028] In yet another embodiment, the invention provides a method of treating a subject by administering an effective amount of stimulating agent which modulates gene expression. The term "modulate" refers to inhibition or suppression of expression when an antimicrobial agent as described herein is over expressed, and induction of expression when an antimicrobial agent as described herein is underexpressed. The term "effective amount" means that amount of stimulating agent which is effective in modulating gene expression of an antimicrobial agent as described herein, resulting in reducing the symptoms of respiratory microbial infection of respiratory tissue. A stimulating agent which modulates gene expression of a response-inhibiting agent as described herein may be a polynucleotide, for example. The polynucleotide may be an antisense, a triplex agent, or a ribozyme. For example, an antisense directed to the structural gene region or to the promoter region of an antimicrobial agent as described herein may be utilized. The stimulating agent which modulates gene expression of an antimicrobial agent as described herein may also be a small interfering RNAs (siRNAs).

[0029] In another embodiment, the invention provides a method for utilizing compounds that modulate activity of an antimicrobial agent as described herein. Compounds that affect activity of an antimicrobial agent as described herein (e.g., antagonists and agonists) include peptides, peptidomimetics, polypeptides, chemical compounds, minerals such as zincs, and biological agents.

[0030] A method for screening for a stimulating agent as defined herein, comprises contacting a respiratory tissue exhibiting respiratory microbial infection, with a candidate compound; and measuring activity in said tissue of an LKKTET or LKKTNT peptide, wherein an increase of activity of said peptide in said tissue, compared to a level of activity of said peptide in a corresponding tissue lacking said candidate compound, indicates that said compound is capable of inducing said stimulating agent.

[0031] A further method of screening for a stimulating agent as defined herein, comprises contacting a respiratory tissue with a candidate compound, optionally microbially infecting the tissue, and measuring LKKTET or LKKTNT peptide activity in said tissue, wherein an increase of activity in said tissue, compared to a level of said LKKTET or LKKTNT peptide activity in a corresponding tissue lacking said candidate compound, indicates that said candidate compound is capable of stimulating production in said tissue of said peptide.

Example 1

[0032] The purpose of this study was to study the antimicrobial activity of peptides against common pathogens *in vitro*. Antimicrobial assays were performed to assess peptide activity against *Pseudomonas aeruginosa*. All tested samples (n=3) constitutively expressed mRNA for thymosin β-4 (Tβ4). This expression was not affected by IL-1β or TNF-α. None of the tested samples expressed hBD-4, -5, -6, HE2β1, histatins (Hist-1, -3), or liver-expressed antimicrobial peptides (LEAP-1, -2). Tβ4 (EC_{50}= 26.5+1.6μg/ml) was effective against *Pseudomonas aeruginosa*. The lowest dose where activity and/or protection was demonstrated against *Pseudomonas aeruginosa* was about 0.5-1 μg/ml.

Example 2

[0033] Antimicrobial assays were performed to assess peptide activity against *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* (PA), and *Staphylococcus epidermidis* (SE).

[0034] Tβ4 (EC_{50} = 18.1 ± 1.7μg/ml) was effective against PA and was weakly effective against staphylococcal strains.

CLAIMS

1. A method of treatment for treating, preventing, inhibiting or reducing respiratory microbial infection of respiratory tissue of a subject, comprising administering to a subject in need of such treatment an effective amount of a composition comprising an antimicrobial agent comprising amino acid sequence LKKTET or LKKTNT, a conservative variant thereof, or a stimulating agent that stimulates production of an LKKTET or LKKTNT peptide, or a conservative variant thereof, in said tissue, so as to inhibit said microbial infection.

2. The method of claim 1 wherein said infection is bacterial infection.

3. The method of claim 1 wherein said infection is by *Pseudomonas aeruginosa*.

4. The method of claim 1 wherein said antimicrobial agent is thymosin beta 4 (Tβ4).

5. The method of claim 1 wherein said antimicrobial agent is other than Tβ4.

6. The method of claim 5 wherein said antimicrobial agent comprises amino acid sequence LKKTET, LKKTNT, KLKKTET, or LKKTETQ, an N-terminal variant of Tβ4, a C-terminal variant of Tβ4, an isoform of Tβ4, or oxidized Tβ4.

7. The method of claim 1 wherein said antimicrobial agent is directly or indirectly antimicrobial.

8. The method of claim 7 wherein said antimicrobial agent is indirectly antimicrobial, and said antimicrobial agent stimulates production of an LKKTET or LKKTNT peptide in tissue of said subject.

9. The method of claim 1 wherein said antimicrobial agent is administered to said subject at a dosage within a range of about 1-30 micrograms.

10. The method of claim 1 wherein said antimicrobial agent is administered by direct administration to said tissue, or by intravenous, intraperitoneal, intramuscular, subcutaneous, inhalation, transdermal or oral administration, to said subject.

11. The method of claim 1 wherein said composition is administered systemically.

12. The method of claim 1 wherein said composition is administered directly to said tissue.

13. The method of claim 12 wherein said composition is in the form of a solution, gel, creme, paste, lotion, spray, suspension, dispersion, salve, hydrogel or ointment formulation.

14. The method of claim 1 wherein said agent is a recombinant or synthetic peptide.

15. A method of treatment for treating, preventing, inhibiting or reducing respiratory microbial infection of respiratory tissue of a subject, comprising administering to a subject in need of such treatment an effective amount of a composition comprising a stimulating agent that stimulates production of an antimicrobial polypeptide comprising amino acid sequence LKKTET or LKKTNT, or a conservative variant thereof, having antimicrobial activity.

16. The method of claim 15 wherein said polypeptide is Thymosin beta 4.

17. The method of claim 15, wherein said infection is bacterial infection.

18. The method of claim 15, wherein said infection is by gram-negative bacteria.

19. The method of claim 15, wherein said infection is by *Pseudomonas aeruginosa*.

20. The method of claim 1, wherein said infection is by gram-negative bacteria.

21. A method for screening for a stimulating agent as defined in claim 1, comprising contacting a respiratory tissue exhibiting respiratory microbial infection, with a candidate compound; and measuring activity in said tissue of an LKKTET or LKKTNT peptide, wherein an increase of activity of said peptide in said tissue, compared to a level of activity of said peptide in a corresponding tissue lacking said candidate compound, indicates that said compound is capable of inducing said stimulating agent.

22. The method of claim 21 wherein said LKKTET peptide is thymosin beta 4.

23. A method of screening for a stimulating agent as defined in claim 1, comprising contacting a respiratory tissue with a candidate compound, and measuring

LKKTET or LKKTNT peptide activity in said tissue, wherein an increase of activity in said tissue, compared to a level of said LKKTET or LKKTNT peptide activity in a corresponding tissue lacking said candidate compound, indicates that said candidate compound is capable of stimulating production in said tissue of said peptide.

24. The method of claim 23, further comprising microbially infecting the tissue, after said contacting.

25. The method of claim 23 wherein said LKKTET peptide is thymosin beta 4.