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WO 2014078900 A1
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(54) Title: AGENTS AND METHODS OF TREATMENT

(57) Abstract: The present disclosure relates generally to a method for controlling pathogen infestation in human and animal subjects and agents and formulations and cells including plant extracts useful for same.



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AGENTS AND METHODS OF TREATMENT

FILING DATA

[0001] This application is associated with and claims priority from Australian Provisional Patent Application No. 2014901480, filed on 24 April 2014, entitled "Agents and methods of treatment", the entire contents of which, are incorporated herein by reference.

BACKGROUND

[0002] The present disclosure relates generally to a method for controlling pathogen infestation in human and animal subjects and agents and formulations and cells including plant extracts useful for same.

FIELD

DESCRIPTION OF RELATED ART

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] Pathogen infestation can lead to significant health issues in humans and animals. These health issues contribute to ever escalating human and animal healthcare expenditure. Even preventative measures require significant fiscal outlays.

[0006] Agricultural losses due to animal including poultry pathogens such as fungal pathogens, for example, are a major problem in the agricultural industry and each year millions of dollars are spent on the topical application by fungicides to curb these losses.

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[0007] Although chemical and antibiotic pathogenicides including fungicides have been successful in human and veterinary medicine, the increasing use of these agents is providing selective pressure for emergence of resistant strains of pathogens. There is clearly a need to develop alternative mechanisms of controlling infestation by human and animal pathogens or to more efficiently manage existing agents.

[0008] Plants have evolved various systems to provide some natural protection against pathogen infestation. These innate immune systems comprise both constitutive or pre-formed and inducible components. Hitherto, there has not been significant recognition of the potential application of the components of plant innate immune systems to non-plant hosts. Examples of these components are small, disulfide-rich proteins which play a large role in both the constitutive and inducible aspects of plant immunity. They can be categorized into families based on their cysteine arrangements and include the thionins, snakins, thaumatin-like proteins, hevein- and knottin-type proteins, lipid transfer proteins, hairpinins and cyclotides as well as defensins.

[0009] Plant defensins are small (45-54 amino acids), basic proteins with four to five disulfide bonds (Janssen *et al.* (2003) *Biochemistry* 42(27):8214-8222). They share a common disulfide bonding pattern and a common structural fold, in which a triple-stranded, antiparallel β -sheet is tethered to an α -helix by three disulfide bonds, forming a cysteine-stabilized $\alpha\beta$ motif. A fourth disulfide bond also joins the N- and C-termini leading to an extremely stable structure. A variety of functions has been attributed to defensins, including anti-bacterial activity, protein synthesis inhibition and α -amylase and protease inhibition (Colilla *et al.* (1990) *FEBS Lett* 270(1-2):191-194; Bloch and Richardson (1991) *FEBS Lett* 279(1):101-104).

[0010] The structure of defensins consists of seven 'loops', defined as the regions between cysteine residues. Loop 1 encompasses the first β -strand (1A) as well as most of the flexible region that connects this β -strand to the α -helix (1B) between the first two invariant cysteine residues. Loops 2, 3 and the beginning of 4 (4A) make up the α -helix, while the remaining loops (4B - 7) make up β -strands 2 and 3 and the flexible region that

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connects them (β -hairpin region) (van der Weerden *et al.* (2013) *Cell Mol Life Sci* 70 (19): 3545-3570). This hairpin region of plant defensins forms a γ -core motif that is found in many anti-microbial peptides of diverse classes (Yount and Yeaman (2005) *Protein Pept Lett* 12(1):49-67).

[0011] Despite their conserved structure, plant defensins share very little sequence identity, with only the eight cysteine residues completely conserved. The cysteine residues are commonly referred to as "invariant cysteine residues", as their presence and location are conserved amongst defensins. Based on sequence similarity, plant defensins can be categorized into different groups. Within each group, sequence homology is relatively high whereas inter-group amino acid similarity is low (van der Weerden *et al.* (2013) *Cell Mol Life Sci* 70 (19): 3545-3570).

[0012] Plant defensins can be divided into two major classes. Class I defensins consist of an endoplasmic reticulum (ER) signal sequence followed by a mature defensin domain. Class II defensins are produced as larger precursors with C-terminal pro-domains or pro-peptides (CTPPs) of about 33 amino acids. Most of the Class II defensins identified to date have been found in *Solanaceous* plant species.

[0013] Class II *Solanaceous* defensins are expressed in floral tissues. They include NaD1, which is expressed in high concentrations in the flowers of ornamental tobacco *Nicotiana glauca* (Lay *et al.* (2003) *Plant Physiol* 131(3):1283-1293). The anti-fungal activity of this peptide involves binding to the cell wall, permeabilization of the plasma membrane and entry of the peptide into the cytoplasm of the hyphae (van der Weerden *et al.* (2008) *J Biol Chem* 283(21):14445-14452) and induction of reactive oxygen species (Hayes *et al.* (2014) *Cell Mol Life Sci*. February 2014, on line ISSN 1420-682X).

[0014] Class II *Solanaceous* defensins have variable degrees of activity against plant fungi. Some Class I defensins exhibit very low anti-fungal activity. Very little research has been conducted hithertofore on the effects of plant defensins on non-plant pathogens.

[0015] Defensins with highly divergent sequences act *via* different mechanism of actions.

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Permeabilization of the plasma membrane is a common feature that is observed for a number of defensins. However, the mechanism of permeabilization and its role in cell death differs between different defensins. Some defensins cause membrane permeabilization at high concentrations, but not at the concentration required for complete growth inhibition. In fact, the concentration of these proteins required to cause significant membrane permeabilization is around 20 times that required for growth inhibition. These proteins do cause slight membrane permeabilization at concentrations required for growth inhibition but only after long time periods (>150 mins). This is likely a result of fungal cell death that occurs after this time. The SYTOX green assay described in US Patent Application No. 12/535,443 has been successfully used to assay permeabilization.

[0016] In contrast, to some other plant defensins, the plant defensin NaD1 causes significant membrane permeabilization at concentrations corresponding to the IC₅₀. Permeabilization by NaD1 begins within 15 minutes and reaches its maximum after 80 minutes (van der Weerden *et al.* (2010) *J Biol Chem* 285(48):37513-37520). NaD1 also causes some membrane permeabilization at low concentrations that do not cause growth inhibition (van der Weerden *et al.* (2008) *J Biol Chem* 283(21):14445-14452). Difference in the permeabilization kinetics between defensins is likely due to differences in the mechanism of action of the proteins. Hence, there is a role in using permeabilization assays to select appropriate defensins.

[0017] There is a need to develop protocols to more effectively manage pathogen infection and infestation in humans and animals. The ability to more efficiently facilitate control of pathogens with reduced application of antibiotic agents or without the need for this application altogether will reduce the potential for resistant strains of pathogens to emerge. This is of particular concern, not only in hospital and healthcare facilities, but also within high density animal facilities.

SUMMARY

[0018] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

[0019] The present disclosure teaches a method for inhibiting growth or infestation of a pathogen in or on a human or animal subject, the method comprising contacting the pathogen with an effective amount of a combination of a plant defensin or a functional natural or synthetic derivative or variant thereof and either: (i) a proteinase inhibitor; or (ii) a chemical pathogenicidic or pathogenistatic agent; in a combined effective amount to inhibit growth or infestation of the pathogen. In an embodiment, the combination of the defensin and the (i) proteinase inhibitor; or (ii) chemical agent; has a synergistic effect compared to the use of each alone at the same individual dose as used in the combination.

[0020] In an embodiment, the plant defensin or its functional natural or synthetic derivative or variant is a permeabilizing defensin. These include Class I defensins and Class II *Solanaceous* defensins. Examples include NaD1 (Q8GTM0), TPP3 (AAA80496), PhD1 (Q8H6Q1), PhD1A (SEQ ID NO:36), PhD2 (Q8H6Q0), FST (p32026), NoD173 (SEQ ID NO:37), HXL001, HXL002, HXL004, HXL007, HXL008, HXL009, HXL012, HXL013, HXL015, HXL035 and HXL036. In an embodiment, the permeabilizing defensin is a functional natural or synthetic derivative or variant thereof. Examples of synthetic variants include where a Loop1B from a Class I defensin replaces the Loop1B from the *Solanaceous* Class II defensin. These include HXP4, HXP34, HXP35, HXP37, HXP58, HXP72, HXP91, HXP92, HXP95 and HXP107.

[0021] Reference to a "proteinase inhibitor" includes a plant proteinase inhibitor or a proteinase inhibitor of non-plant origin as well as a precursor form which requires to be processed into an active form. Where a proteinase inhibitor is used in generating a formulation with a defensin, any precursor form is activated prior to use. Examples of proteinase inhibitors include serine and cysteine proteinase inhibitors such as NaCys1,

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NaCys2, NaCys3, NaCys4, HvCPI6, SlCys9, Oc-Ia, Oc-Ib, Oc-Ic, Oc-Id, StPin1A, At2g38870, CI-1B, CI-2, At2g43510 and BPTI.

[0022] Reference to a "chemical agent" includes a proteinaceous and non-proteinaceous chemical molecule. Chemical pathogenicidic or pathogenistatic agents include a fungicide selected from the list consisting of ciclopirox, terbinafine, fenpropimorph, ketoconazole, intraconazole, fluconazole, amorolfine, amphotericin, azole, polyene, echinocandin, allylamine, griseofulvin, tolnaftate, benzoxaborole, aganocide, flucytosine, haloprogin, polygodial and undecylenic acid. An example of an echinocandin fungicide is caspofungin. Chemical cell wall synthase inhibitors are also contemplated herein. Proteinaceous pathogenicidic or pathogenistatic agents include a β -glucan synthase inhibitor and a chitin synthase inhibitor as well as other cell wall synthase inhibitors.

[0023] It is surprisingly determined herein that the combination of a plant defensin and one of a proteinase inhibitor or a chemical pathogenicidic or pathogenostatic agent facilitates anti-pathogen activity. The anti-pathogen activity of each of the defensin, proteinase inhibitor and chemical agent is enhanced when the defensin is used in combination with either of the other two agents. In an embodiment, the enhancement results from a synergistic interaction between the defensin and either the proteinase inhibitor or chemical agent.

[0024] The pathogen includes a fungus. A fungus includes a yeast. In an embodiment, the pathogen is a human or animal fungal pathogen. Animal including mammalian such as human fungal pathogens include species of *Alternaria* spp, *Aspergillus* spp, *Candida* spp, *Fusarium* spp, *Trichophyton* spp, *Cryptococcus* spp, *Histoplasma* spp, *Microsporum* spp, *Penicillium* spp, *Pneumocystis* spp, *Trichosporon* spp, *Scedosporium* spp, *Paecilomyces* spp, *Acremonium* spp, *Stachybotrys* spp and Dermatiaceous molds. Specific animal, including mammalian and in particular human pathogens include *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus paraciticus*, *Candida albicans*, *Candida dubliniensis*, *Candida famata*, *Candida glabrata*, *Candida guilliermondii*, *Candida haemulonii*, *Candida kefyr*, *Candida krusei*, *Candida lusitaniae*, *Candida norvegensis*, *Candida parapsilosis*, *Candida*

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tropicalis, *Candida viswanathii*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium moniliforme*, *Trychophyton rubrum*, *Trychophyton mentagrophytes*, *Trychophyton interdigitales*, *Trychophyton tonsurans*, *Cryptococcus neoformans*, *Cryptococcus gattii*, *Cryptococcus grubii*, *Microsporum canis*, *Microsporum gypseum*, *Penicillium marneffei*, *Trichosporon beigeli*, *Trichosporon asahii*, *Trichosporon inkin*, *Trichosporon asteroides*, *Trichosporon cutaneum*, *Trichosporon domesticum*, *Trichosporon mucoides*, *Trichosporon ovoides*, *Trichosporon pullulans*, *Trichosporon loubieri*, *Trichosporon japonicum*, *Scedosporium apiospermum*, *Scedosporium prolificans*, *Paecilomyces variotii*, *Paecilomyces lilacinus*, *Acremonium strictum*, *Cladophialophora bantiana*, *Wangiella dermatitidis*, *Ramichloridium obovoideum*, *Chaetomium atrobrunneum*, *Dactylaria gallopavum*, *Bipolaris* spp, *Exserohilum rostratum* as well as *Absidia corymbifera*, *Apophysomyces elegans*, *Mucor indicus*, *Rhizomucor pusillus*, *Rhizopus oryzae*, *Cunninghamella bertholletiae*, *Cokeromyces recurvatus*, *Saksenaea vasiformis*, *Syncephalastrum racemosum*, *Basidiobolus ranarum*, *Conidiobolus coronatus*/*Conidiobolus incongruus*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Pseudallescheria boydii* and *Sporothrix schenckii*.

[0025] The defensin and either the proteinase inhibitor or chemical agent may be topically applied to the human or animal or systemically administered to the human or animal. The defensin and/or proteinase inhibitor may also be provided as part of a cellular extract which includes a plant extract. This is useful, for example, in herbal formulations such as body and hair washes.

[0026] Further taught herein is a formulation comprising both the defensin and either the proteinase inhibitor or chemical agent or a combination of formulations each comprising one of the defensin or proteinase inhibitor or chemical agent. The formulations are then combined prior to or during use. Reference to a "formulation" includes a cell or plant extract.

[0027] Enabled herein is a use of a plant defensin and either the proteinase inhibitor or chemical agent in the manufacture of a medicament for the treatment or prophylaxis of

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pathogen infestation of a human or animal.

[0028] Humans which can be treated include a human of any age. Animals which may be treated include farm animals, companion animals, laboratory test animals and wild animals.

[0029] A kit comprising compartments each containing a plant defensin or a functional natural or synthetic derivative or variant thereof, and one or both of a proteinase inhibitor and/or a chemical agent each in separate compartments, is also taught herein. Assays to identify suitable defensins and optimizing concentrations of various components are also contemplated by the present disclosure.

[0030] In another embodiment, microorganisms belonging to genera or species of normal flora are genetically engineered to produce a protein such as a defensin and/or a proteinase inhibitor. Such microorganisms may be used as a probiotic to colonize gut regions or skin surface regions which may be used in conjunction with an anti-fungal chemical agent. Alternatively, the defensin and/or proteinase inhibitor is provided as a cell extract including a plant.

Table 1
Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Amino acid sequence of C-terminal end amino acid sequence of NaD1 which ends and includes the most C-terminal invariant cysteine residue
2	A modified amino acid sequence of Class II <i>Solanaceous</i> defensin Loop1B region
3	A modified amino acid sequence of Class II <i>Solanaceous</i> defensin Loop1B region
4	A modified amino acid sequence of Class II <i>Solanaceous</i> defensin Loop1B region
5	A modified amino acid sequence of Class II <i>Solanaceous</i> defensin Loop1B region
6	A modified amino acid sequence of Class II <i>Solanaceous</i> defensin Loop1B region
7	Amino acid sequence of Loop1B from NaD1
8	Amino acid sequence of HXP4 protein
9	Amino acid sequence of full length HXL001 protein
10	Amino acid sequence of full length HXL002 protein
11	Amino acid sequence of full length HXL004 protein
12	Amino acid sequence of full length HXL007 protein
13	Amino acid sequence of full length HXL008 protein
14	Amino acid sequence of full length HXL013 protein
15	Amino acid sequence of HXP34 protein
16	Amino acid sequence of HXP35 protein
17	Amino acid sequence of HXP37 protein
18	Amino acid sequence of HXP58 protein
19	Amino acid sequence of HXP72 protein
20	Amino acid sequence of HXP91 protein
21	Amino acid sequence of HXP92 protein

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SEQUENCE ID NO:	DESCRIPTION
22	Amino acid sequence of HXP95 protein
23	Amino acid sequence of HXP107 protein
24	Amino acid sequence of full length HXL015 protein
25	Amino acid sequence of mature HXL012 protein
26	Amino acid sequence of mature HXL001 protein
27	Amino acid sequence of mature HXL002 protein
28	Amino acid sequence of mature HXL004 protein
29	Amino acid sequence of mature HXL007 protein
30	Amino acid sequence of mature HXL008 protein
31	Amino acid sequence of mature HXL013 protein
32	Amino acid sequence of mature HXL015 protein
33	Amino acid sequence of mature HXL009 protein
34	Amino acid sequence of mature HXL035 protein
35	Amino acid sequence of mature HXL036 protein
36	Amino acid sequence of mature PhD1A protein
37	Amino acid sequence of mature NoD173 protein

DETAILED DESCRIPTION

[0031] Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any element or integer or method step or group of elements or integers or method steps.

[0032] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a defensin" includes a single defensin, as well as two or more defensins; reference to "an agent" includes a single agent, as well as two or more agents; reference to "the disclosure" includes single and multiple aspects taught by the disclosure; and so forth. Aspects taught and enabled herein are encompassed by the term "invention". All such aspects are enabled within the width of the present invention.

[0033] A protocol is developed to facilitate management of pathogen infestation in human and animal subjects. The protocol comprises the use of a plant defensin or a functional natural or synthetic derivative or variant thereof in combination with either a proteinase inhibitor or a chemical pathogenocidic or pathogenostatic agent. It is proposed herein that the plant defensin and the proteinase inhibitor or chemical agent enable an efficacious treatment and prophylaxis protocol against pathogens which infect a human or animal subject. In an embodiment, the effect of the defensin and one or other of the proteinase inhibitor or chemical agent is synergistic.

[0034] Hence, enabled herein is a method for inhibiting growth or infestation of a pathogen in a human or animal subject, the method comprising contacting the pathogen with an effective amount of a combination of a plant defensin or a functional natural or synthetic derivative or variant thereof and either: (i) a proteinase inhibitor; or (ii) a chemical pathogenocidic or pathogenostatic agent; in a combined effective amount to inhibit growth or infestation of the pathogen. As indicated above, in an embodiment, the combination of the defensin and the proteinase inhibitor or chemical agent is synergistic

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compared to the use of each alone at the same individual dose as used in the combination. For convenience, a human and animal subject may also be referred to as a "host", "individual", "target", "recipient" or "patient". The defensin, proteinase inhibitor and chemical entity may each be referred to as an "agent" or collectively as "agents". The agents may be in purified form or comprised within a cell or plant extract. In an embodiment, the chemical entity is added to the cell or plant extract.

[0035] In an embodiment, the inhibitory effect of a given defensin, proteinase inhibitor or chemical pathogenicide or pathogenostatic agent alone is significantly enhanced when the defensin is used together with the proteinase inhibitor or the chemical agent. In an embodiment, therefore, the combination is synergistic. Greco *et al.* (1995) *Pharmacol Rev.* 47:331-385 define a category of synergy on the basis that the use of two agents in combination has greater activity relative to the additive effects when each is assayed alone. Hence, the definition adopted herein includes all such situations provided that the combined effect of the two agents acting together is greater than the sum of the individual agents acting alone. Furthermore, a combination of agents is deemed synergistic, as the term is intended herein, if there exists a set of conditions, including but not limited to concentrations, where the combined effect of the agents acting together is greater than the sum of the individual components acting alone. Richer (1987) *Pestic Sci* 19:309-315 describes a mathematical approach to establish proof of synergy. This approach uses Limpel's formula for comparing an observed level of inhibition (I_o) in the combined presence of two inhibitor agents, X and Y, with an expected additive effect (E_e) resulting from each X or Y acting separately at the same respective concentrations as used to measure their combined effect. Additive percent inhibition, E_e , is calculated as $X + Y - XY/100$ where X and Y are expressed as percent inhibition. Synergism exists where $I_o > E_e$.

[0036] Synergy may be expressed as a synergy scale. In an embodiment, a value of up to 15 represents no significant synergy such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14; a value of from 15 up to 30 represents low synergy such as 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29; a value of from 30 to 60 represents medium synergy such as 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53,

54, 55, 56, 57, 58, 59 or 60; a value greater than 60 represents a high degree of synergy. By "greater than 60" includes from 61 to 100 including 61, 70, 80, 90 and 100 and any value in between.

[0037] As indicated above, a "synergistic effect" occurs where two or more agents within the disclosed protocol produce a combined effect that is greater than the sum of the individual effects of each agent acting alone. The effect may be one or more of efficacy, stability, rate, and/or level of toxicity. As described herein, inhibition of pathogen growth is considered synergistic when, measured in the combined presence of at least one plant defensin and at least one of a proteinase inhibitor or a chemical agent is greater than the summed inhibition measured in the presence of a particular concentration range of each agent, defensin and either the proteinase inhibitor or chemical agent, individually, under otherwise identical conditions. It will be understood that it is not necessary that a greater than additive effect be observed with every combination of concentrations of the two agents in order to be deemed synergistic. The synergistic effect of the two agents can be observed under certain concentration combinations, but not in others. For example, if entry into the pathogen limits toxicity, the presence of defensin can result in synergy, especially if the concentration of the proteinase inhibitor or chemical agent is sub-maximal with respect to inhibition. In one embodiment, the concentration of one or both of the defensin and the proteinase inhibitor or chemical agent is sub-maximal. By the same token, synergy can be masked if one or two of the agents is present at such a high level (maximum level) as to result in maximum observable inhibition. The general system for a defensin-proteinase inhibitor/chemical agent combination is, therefore, termed "synergistic" because the potential for synergy is present even if synergy is not observed under all conditions. The synergy between a plant defensin and the proteinase inhibitor or chemical agent provides greater pathogen inhibition than can be obtained by individual agents acting alone, for at least some dosages. In some cases, one of the defensin or other agent is not measurably effective against a particular pathogen until combined with the other. Therefore, the present invention provides for increased protection of a host from pathogen infestation with reduced dependence on antibiotics or chemical agents. This means decreased input cost to the human and animal healthcare system, a broader spectrum of activity against pathogens and reduced potential for spread of resistance. Hence, in

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relation to the latter, the selection pressure for development of pathogenicide-resistant pathogen strains is greatly reduced, which allows for an extended commercial life of the agents as well as reduced proliferation of resistant pathogens and reduced likelihood of emergence of multiple-resistant strains of pathogens.

[0038] "Pathogen inhibition" includes both pathogenicidic and pathogenostatic activity, as measured by reduction of pathogen growth (or loss of viability) compared to a control. Pathogen growth can be measured by many different methods known in the art depending on the pathogen. A commonly used method of measuring growth of a filamentous fungus, for example, entails germinating spores in a suitable growth medium, incubating for a time sufficient to achieve measurable growth, and measuring increased optical density in the culture after a specified incubation time. The optical density rises with increased growth. Typically, fungal growth is necessary for pathogenesis. Therefore, inhibition of pathogen growth provides a suitable indicator for protection from fungal disease, i.e. the greater the inhibition, the more effective the protection.

[0039] "Preventing infection" in the present context, means that the human or animal with the combination of defensin and proteinase inhibitor or chemical agent avoids pathogen infection or disease symptoms or all of the above, or exhibits reduced or minimized or less frequent pathogen infection or disease symptoms or all of the above, that are the natural outcome of the host-pathogen interactions when compared to the host not exposed to the defensin, the combination of the defensin and either the proteinase inhibitor or the chemical agent. That is to say, pathogens are prevented or reduced from causing disease and/or the associated disease symptoms. Infection and/or symptoms are reduced by at least about 10%, 20%, 30%, 40%, 50, 60%, 70% or 80% or greater as compared to a host not so treated with the protocol taught herein. The percentage reduction can be determined by any convenient means appropriate to the host and pathogen.

[0040] Hence, the combined action of the defensin and either proteinase inhibitor or chemical agent is to inhibit pathogen growth, replication, infection and/or maintenance, amongst other inhibitory activities and/or induces amelioration of symptoms of pathogen infestation.

[0041] Human and animal protection (disease prophylaxis or treatment) can initially be tested using *in vitro* laboratory assays (e.g. pathogen inhibition assays) followed by animal studies and eventually human clinical trials.

[0042] By "contacting" includes exposure of the pathogen to the combination of the defensin and either the proteinase inhibitor or chemical agent following topical or systemic administration or application to the human or animal subject. Hence, the defensin and either the proteinase inhibitor or chemical agent may be applied topically to a surface area on the human or animal subject or they may be systemically administered to the human or animal subject. In addition, some components such as a defensin and/or the proteinase inhibitor can be produced by microorganisms. In another embodiment, microorganisms belonging to genera or species of normal flora are genetically engineered to produce a protein such as a defensin and/or a proteinase inhibitor. Such microorganisms may be used as a probiotic to colonize gut regions or skin surface regions which may be used in conjunction with an anti-fungal chemical agent. Alternatively, the defensin and/or proteinase inhibitor is provided as a cell extract which includes a plant extract. The plant may naturally produce the defensin or proteinase inhibitor or both or it may be engineered to produce one or other or both.

[0043] In an embodiment, the defensin and either the proteinase inhibitor or chemical agent are formulated together such as in a topical formulation, hair or body washing solution or a formulation suitable for systemic administration to the appropriate host. Topical formulations include an aqueous solution, a liquid formulation, a tonic, eye drops, ear drops, a wash, a spray, paint, powder, dispersant, an atomized formulation, a douche, cream, ointment, lipstick, gel, sludge, paste, patch, impregnated bandage and the like. A formulation comprising a cell or plant extract comprising a defensin and optionally a proteinase inhibitor is contemplated herein. A fungicide may also be added.

[0044] Enabled herein is a formulation comprising a plant defensin or a functional natural or synthetic derivative or variant thereof and either a proteinase inhibitor or chemical pathogenicidic or pathogenostatic agent for use in inhibiting growth or infestation of a

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pathogen in or on a human or animal subject.

[0045] In an embodiment, taught herein is a therapeutic kit comprising multiple compartments wherein a first compartment comprises a plant defensin or a functional natural or synthetic derivative or variant thereof, a second compartment comprises one of proteinase inhibitor or a chemical pathogenocidic or pathogenostatic agent and optionally a third or further compartment comprising excipients, carriers or diluents wherein in use the contents of the first and second compartments are admixed prior to or during application to a human or animal subject or a surface. In an embodiment, all three of a defensin, proteinase inhibitor and a chemical agent are included in separate containers. Alternatively, the kit comprises microorganisms in freeze dried or other reconstructable forms which are engineered to express a defensin and/or a proteinase inhibitor. The kit may then contain a compartment comprising a chemical agent having anti-pathogen properties. The defensin and/or proteinase inhibitor may also be purified from a particular source or is within a plant cell extract.

[0046] Described and enabled herein is a defensin or a functional natural or synthetic derivative or variant thereof and either a proteinase inhibitor or chemical agent having anti-pathogen properties for use in inhibiting growth or infestation of a pathogen in or on a human or animal subject, the defensin and other agent being used in combination so as to act to facilitate pathogen inhibition. In an embodiment, the combination is synergistic.

[0047] Reference to a "plant defensin" includes a functional natural or synthetic derivative or variant thereof unless the context clearly indicates otherwise. Examples of suitable defensins contemplated herein include permeabilizing defensins, *Solanaceous* Class II defensins and functional natural or synthetic derivatives or variants thereof.

[0048] The defensins used herein may be referred to herein as "naturally occurring" defensin, a "modified" defensin, a "variant" defensin, a "mutated" defensin or a "chimeric" defensin, depending on its source.

[0049] In an embodiment, the permeabilizing defensin is a Class II *Solanaceous* defensin.

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In an embodiment, the defensin is modified at the loop region between the first β -strand (β -strand 1) and the α -helix at the N-terminal end portion of the defensin. In an embodiment, the loop region comprises the 6 amino acids N-terminal of the second invariant cysteine residue or its equivalent. This region is defined as "Loop1B". A Class II *Solanaceous* defensin is distinguished from other defensins by a relatively conserved C-terminal end portion of the mature domain. Reference to a "Class II *Solanaceous* defensin" includes any defensin having at least 70% amino acid sequence similarity to the C-terminal end portion of the NaD1 mature domain, the C-terminal portion of NaD1 comprising approximately 20 contiguous amino acid residues ending and including the most C-terminal invariant cysteine in the NaD1 mature domain (for example, SEQ ID NO:1). By "at least 70%" means at least 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%.

[0050] In an embodiment, the Loop1B amino acid sequence in a Class II *Solanaceous* defensin is modified to the sequence $X_1 X_2 X_3 X_4 X_5 X_6$ (SEQ ID NO:2) wherein:

X_1 is A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V or a naturally occurring modified form thereof;

X_2 is A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V or a naturally occurring modified form thereof;

X_3 is A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V or a naturally occurring modified form thereof;

X_4 is A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V or a naturally occurring modified form thereof;

X_5 is A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V or a naturally occurring modified form thereof; and/or

X_6 is A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V or a naturally occurring modified form thereof;

using single letter amino acid nomenclature, wherein the amino acid sequence $X_1 X_2 X_3 X_4 X_5 X_6$ does not correspond to an amino acid sequence of the Loop1B region from the Class II *Solanaceous* defensin prior to modification.

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[0051] In an embodiment, the Loop1B sequence in a Class II *Solanaceous* defensin is modified to the sequence X₁ X₂ X₃ X₄ X₅ X₆ (SEQ ID NO:3) wherein:

X₁ is N, G, D, H, K, A, E, Q, T, P, L, M, S, or R;

X₂ is K, R, G, H, L, N, F, I, S, T or Y;

X₃ is W, Y, H, L, G, F or P;

X₄ is P, K, S, R, H, T, E, V, N, Q, D or G;

X₅ is S, K, Y, F, G or H; and/or

X₆ is P, V, L, T, A, F, N, K, R, M, G, H, I or Y;

wherein the amino acid sequence X₁ X₂ X₃ X₄ X₅ X₆ does not correspond to an amino acid sequence of the Loop1B region from the Class II *Solanaceous* defensin prior to modification.

[0052] In an embodiment, the Loop1B sequence in a Class II *Solanaceous* defensin is modified to the sequence X₁ X₂ X₃ X₄ X₅ X₆ (SEQ ID NO:4) wherein:

X₁ is N, H, Q, D, K or E;

X₂ is R, H, T, K or G;

X₃ is F, H, Y or W;

X₄ is P, K, S or R;

X₅ is G or F; and

X₆ is P, V, I or N;

wherein the amino acid sequence X₁ X₂ X₃ X₄ X₅ X₆ does not correspond to an amino acid sequence of the Loop1B region from the Class II *Solanaceous* defensin prior to modification.

[0053] Reference to "X₁ X₂ X₃ X₄ X₅ X₆" means 6 contiguous amino acid residues corresponding to a Loop1B region.

[0054] In an embodiment, the artificially created or modified defensin comprises the amino acid sequence as set forth in SEQ ID NO:5. In this sequence, the Loop1B region is defined as X₁ X₂ X₃ X₄ X₅ X₆ wherein:

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X₁ is an amino acid selected from the list consisting of: L, F, S, I, A, H, Y, Q, D, K or G;

X₂ is an amino acid selected from the list consisting of: S, V, F, I, K, L, A, P, N, T, R, H or G;

X₃ is an amino acid selected from the list consisting of: A, F, W, N, I, S, Y, P, L or H;

X₄ is an amino acid selected from the list consisting of: K, G, E, R, A, P, F, Q, V or S;

X₅ is an amino acid selected from the list consisting of: M, G, K, D, S, Y, P, E, N or F; and

X₆ is an amino acid selected from the list consisting of: V, T, M, S, W, A, P, G, E, K, L, H, I or N.

[0055] In an embodiment, the artificially created or modified defensin comprises the amino acid sequence as set forth in SEQ ID NO:6. In this sequence, the Loop1B region is defined as X₁ X₂ X₃ X₄ X₅ X₆ wherein:

X₁ is an amino acid selected from the list consisting of: N, H, Q, D, K or E;

X₂ is an amino acid selected from the list consisting of: R, H, T, K or G;

X₃ is an amino acid selected from the list consisting of: F, H, Y or W;

X₄ is an amino acid selected from the list consisting of: P, K, S or R;

X₅ is an amino acid selected from the list consisting of: G or F; and

X₆ is an amino acid selected from the list consisting of: P, V, I or N.

[0056] In the case of NaD1, a Class II *Solanaceous* defensin, the Loop1B amino acid sequence is NTFPGI (SEQ ID NO:7). Consequently, the NTFPGI is modified such that N is replaced by one of A, R, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V or a naturally occurring modified form thereof; the T is replaced by one of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, W, Y or V or a naturally occurring modified form thereof; the F is replaced by one of A, R, N, D, C, Q, E, G, H, I, L, K, M, P, S, T, W, Y or V or a naturally occurring modified form thereof; the P is replaced by one of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, S, T, W, Y or V or a naturally occurring modified form thereof; the G is replaced by one of A, R, N, D, C, Q, E, H, I, L, K, M, F, P, S, T, W, Y or V or a

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naturally occurring modified form thereof; and/or the I is replaced by one of A, R, N, D, C, Q, E, G, H, L, K, M, F, P, S, T, W, Y or V; with the proviso that the Loop1B amino acid sequence does not correspond to the Loop1B from NaD1. The Loop1B sequence may have a single amino acid change or 2 or 3 or 4 or 5 or all 6 amino acids may be altered.

[0057] The Class II Solanaceous defensin may be modified by any number of amino acid changes to the Loop1B region alone or in combination with other mutations. Other mutations include amino acid substitutions, additions and/or deletions. Mutations outside the Loop1B region may number from 1 to about 50. A "change" includes a graft of a Loop1B region from one defensin onto a Class II *Solanaceous* defensin Loop1B region. The source may be a Class I defensin Loop1B or a Loop1B from another Class II defensin. These aspects are based on the proviso that the enhanced anti-pathogen activity of the combined use of the modified defensin and other agent against at least one human or animal pathogen is maintained. In an embodiment, the anti-pathogen activity is enhanced relative to the Class II defensin prior to modification in terms of level or spectrum of activity, stability and/or permeabilization. In an embodiment, the anti-pathogen activity is enhanced relative to the peptide prior to modification of the Class II defensin in terms of level or spectrum of activity, stability and/or permeabilization.

[0058] Hence, included herein is the use of a proteinase inhibitor or a chemical agent with anti-pathogen properties with an artificially created defensin comprising a modified Class II *Solanaceous* defensin backbone wherein the loop region between β -strand 1 and the α -helix on the N-terminal end portion is modified by a single or multiple amino acid substitution, addition and/or deletion to generate a variant defensin which has anti-pathogen activity. In an embodiment, the loop region is Loop1B defined by the 6 amino acid residues N-terminal to the second invariant cysteine residue. Its equivalent region in any defensin is contemplated herein.

[0059] Examples of suitable defensins include NaD1, TPP3, PhD1, PhD1A, PhD2, FST, NoD173, HXL001 (SEQ ID NO:26), HXL002 (SEQ ID NO:27), HXL004 (SEQ ID NO:28), HXL007 (SEQ ID NO:29), HXL008 (SEQ ID NO:30), HXL009 (SEQ ID NO:33), HXL012 (SEQ ID NO:25), HXL013 (SEQ ID NO:31), HXL015 (SEQ ID NO:32),

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HXL035 and HXL036. Examples of synthetic defensin variants include HXP4 (SEQ ID NO:8), HXP34 (SEQ ID NO:15) and HXP35 (SEQ ID NO:16). Other examples of variant defensins include HXP37 (SEQ ID NO:17), HXP58 (SEQ ID NO:18), HXP72 (SEQ ID NO:19), HXP91 (SEQ ID NO:20), HXP92 (SEQ ID NO:21), HXP95 (SEQ ID NO:22) and HXP107 (SEQ ID NO:23).

[0060] Taught herein is a method for inhibiting growth or infestation of a pathogen, the method comprising contacting the pathogen with an effective amount of a combination of a plant defensin selected from the list consisting of NaD1, TPP3, PhD1, PhD1A, PhD2, FST, NoD173, HXL001, HXL002, HXL004, HXL007, HXL008, HXL009, HXL012, HXL013, HXL015, HXL035 and HXL036 or a variant or derivative of a defensin selected from the list consisting of HXP4, HXP34, HXP35, HXP37, HXP58, HXP72, HXP91, HXP92, HXP95 and HXP107 and a proteinase inhibitor or chemical agent having anti-pathogen properties, the combination of the defensin and either a proteinase inhibitor or chemical agent facilitates anti-pathogen activities compared to the use of each alone at the same individual dose as used in the combination.

[0061] In an embodiment, the agents act in synergy.

[0062] Suitable proteinase inhibitors include serine and cysteine proteinase inhibitors such as NaPI, NaCys1, NaCys2, NaCys3, NaCys4, HvCPI6, SlCys9, Oc-Ia, Oc-Ib, Oc-Ic, Oc-Id, StPin1A, At2g38870, CI-1B, CI-2, At2g43510 and BPTI.

[0063] Reference to a "chemical agent" includes a proteinaceous and non-proteinaceous chemical molecule. Chemical pathogenicidic or pathogenistatic agents include a fungicide selected from the list consisting of ciclopirox, terbinafine, fenpropimorph, ketoconazole, intraconazole, fluconazole, amorolfine, amphotericin, azole, polyene, echinocandin, allylamine, griseofulvin, tolnaftate, benzoxaborole, aganocide, flucytosine, haloprogin, polygodial and undecylenic acid. An example of an echinocandin fungicide is caspofungin. Chemical cell wall synthase inhibitors are also contemplated herein. Proteinaceous pathogenicidic or pathogenistatic agents include a β -glucan synthase inhibitor and a chitin synthase inhibitor as well as other cell wall synthase inhibitors.

[0064] Taught herein is a method for inhibiting growth or infestation of a pathogen, the method comprising contacting the pathogen with an effective amount of combination of a plant defensin selected from the list consisting of NaD1, TPP3, PhD1, PhD1A, PhD2, FST, NoD173, HXL001, HXL002, HXL004, HXL007, HXL008, HXL009, HXL012, HXL013, HXL015, HXL035 and HXL036 or a variant or derivative of a defensin selected from the list consisting of HXP4, HXP34, HXP35, HXP37, HXP58, HXP72, HXP91, HXP92, HXP95 and HXP107 and either a proteinase inhibitor selected from the list consisting of NaPI, NaCys1, NaCys2, NaCys3, NaCys4, HvCPI6, SlCys9, Oc-Ia, Oc-Ib, Oc-Ic, Oc-Id, StPin1A, At2g38870, CI-1B, CI-2, At2g43510 and BPTI or a chemical agent selected from the list consisting of ciclopirox, terbinafine, fenpropimorph, ketoconazole, intraconazole, fluconazole, amorolfine, amphotericin, an azole, a polyene, an echinocandin (e.g. caspofungin), an allylamine, griseofulvin, tolnaftate, benzoxaborole, aganocide, flucytosine, haloprogin, polygodial, undecylenic acid, a β -glucan synthase inhibitor and a chitin synthase inhibitor the combination of the defensin and the other agent facilitates anti-pathogen activity compared to the use of each alone at the same individual dose as used in the combination. In an embodiment, the combination is synergistic.

[0065] In an embodiment the pathogen is a fungus.

[0066] Taught herein is a method for inhibiting growth or infestation of a fungal pathogen, the method comprising contacting the fungal pathogen with an effective amount of combination of a plant defensin selected from the list consisting of NaD1, TPP3, PhD1, PhD1A, PhD2, FST, NoD173, HXL001, HXL002, HXL004, HXL007, HXL008, HXL009, HXL012, HXL013, HXL015, HXL035 and HXL036 or a variant or derivative of a defensin selected from the list consisting of HXP4, HXP34, HXP35, HXP37, HXP58, HXP72, HXP91, HXP92, HXP95 and HXP107 and either a proteinase inhibitor selected from the list consisting of NaPI, NaCys1, NaCys2, NaCys3, NaCys4, HvCPI6, SlCys9, Oc-Ia, Oc-Ib, Oc-Ic, Oc-Id, StPin1A, At2g38870, CI-1B, CI-2, At2g43510 and BPTI or a chemical agent selected from the list consisting of ciclopirox, terbinafine, fenpropimorph, ketoconazole, intraconazole, fluconazole, amorolfine, amphotericin, an azole, a polyene, an echinocandin (e.g. caspofungin), an allylamine, griseofulvin, tolnaftate, benzoxaborole,

aganocide, flucytosine, haloprogin, polygodial, undecylenic acid, a β -glucan synthase inhibitor and a chitin synthase inhibitor in a combination of the defensin and the other agent which facilitates inhibition or growth of the pathogen. In an embodiment, the combination is synergistic.

[0067] The present method is useful in the treatment or prophylaxis of a subject. The term "subject" includes a human of any age or an animal such as a farm animal (e.g. sheep, pig, horse, cow, donkey, llama, alpaca or poultry bird (e.g. chicken, duck, turkey, pheasant, peacock)), companion animal (e.g. dog or cat), laboratory test animal (e.g. mouse, rat, rabbit, guinea pig or hamster) or captive wild animal (e.g. kangaroo, Tasmanian devil or wild feline animal). A "human" or "animal" includes a part thereof such as fingers, toes, nails, eyes, ears, mouth, nose, sinuses, hooves, reproductive tissues, lungs, skin and scalp.

[0068] Animal including mammalian such as human fungal pathogens include species of *Alternaria* spp, *Aspergillus* spp, *Candida* spp, *Fusarium* spp, *Trichophyton* spp, *Cryptococcus* spp, *Histoplasma* spp, *Microsporum* spp, *Penicillium* spp, *Pneumocystis* spp, *Trichosporon* spp, *Scedosporium* spp, *Paecilomyces* spp, *Acremonium* spp, *Stachybotrys* spp and Dermatiaceous molds. Specific animal, including mammalian and in particular human pathogens include *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus paraciticus*, *Candida albicans*, *Candida dubliniensis*, *Candida famata*, *Candida glabrata*, *Candida guilliermondii*, *Candida haemulonii*, *Candida kefyr*, *Candida krusei*, *Candida lusitanae*, *Candida norvegensis*, *Candida parapsilosis*, *Candida tropicalis*, *Candida viswanathii*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium monoliforme*, *Trychophyton rubrum*, *Trychophyton mentagrophytes*, *Trychophyton interdigitales*, *Trychophyton tonsurans*, *Cryptococcus neoformans*, *Cryptococcus gattii*, *Cryptococcus grubii*, *Microsporum canis*, *Microsporum gypseum*, *Penicillium marneffeii*, *Trichosporon beigeli*, *Trichosporon asahii*, *Trichosporon inkin*, *Trichosporon asteroides*, *Trichosporon cutaneum*, *Trichosporon domesticum*, *Trichosporon mucoides*, *Trichosporon ovoides*, *Trichosporon pullulans*, *Trichosporon loubieri*, *Trichosporon japonicum*, *Scedosporium apiospermum*, *Scedosporium prolificans*, *Paecilomyces variotii*, *Paecilomyces lilacinus*, *Acremonium strictum*, *Cladophialophora bantiana*, *Wangiella dermatitidis*, *Ramichloridium obovoideum*, *Chaetomium*

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atrobrunneum, *Dactlaria gallopavum*, *Bipolaris* spp, *Exserohilum rostratum* as well as *Absidia corymbifera*, *Apophysomyces elegans*, *Mucor indicus*, *Rhizomucor pusillus*, *Rhizopus oryzae*, *Cunninghamella bertholletiae*, *Cokeromyces recurvatus*, *Saksenaea vasiformis*, *Syncephalastrum racemosum*, *Basidiobolus ranarum*, *Conidiobolus coronatus*/*Conidiobolus incongruus*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Pseudallescheria boydii* and *Sporothrix schenckii*.

[0069] The combination of the defensin and either the proteinase inhibitor or chemical agent taught herein is useful for combating pathogen diseases or infection in humans and animals. Hence, the subject specification teaches a protocol for the treatment or prophylaxis of pathogens. The protocol has human and veterinary applications. Further provided is a process of combating pathogens whereby they are exposed to the combination of a defensin and either a proteinase inhibitor or a chemical agent having anti-pathogen properties herein described. One or both of the defensins and/or peptide may be used in the form of a composition.

[0070] Another aspect taught herein is a composition comprising a plant defensin or a functional natural or synthetic derivative or variant thereof and either a proteinase inhibitor or a chemical agent having anti-pathogen properties together with one or more pharmaceutically or veterinary acceptable carriers, diluents or excipients. In another embodiment, two compositions are used, one comprising the defensin and one comprising the other agent. In an embodiment, the composition is in the form of a spray, mist, micro- or nano-particles, an aqueous solution, a wash, a tonic, a dispersant, an atomized formulation, douche, lipstick, sludge, powder, cream, ointment, gel, patch, impregnated bandage, liquid, formulation, paint or other suitable distribution medium including topical or systemic forms of the composition. By "systemic form" includes a form suitable or oral, intravenous intra peripheral, subcutaneous, intrathecal, intracranial, vaginal or rectal administration. A composition includes a cell or plant extract comprising one or other or both of a defensin and/or proteinase inhibitor. A chemical agent may be added to the composition.

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[0071] For compositions comprising the defensin and the other agent described herein, generally include a carrier, excipient, diluent, preservative, stabilizer and/or a solid or liquid additive.

[0072] The composition may take a wide variety of forms depending on the intended method of administration. Generally, but not exclusively, topical compositions are used for human or animal subjects. In preparing the compositions, usual media may be employed such as, for example, water, glycols, oils, alcohols, preservatives and/or coloring agents. The compositions may take the form of a liquid preparation such as, for example, suspensions, elixirs and solutions. Carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like may also be used. The composition may also be in the form of a powder, capsule and tablet.

[0073] When administered by aerosol or spray, the compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons and/or other solubilizing or dispersing agents known in the art.

[0074] The effective dosage of the defensin and other agent may vary depending on the particular combination employed, the mode of administration, the pathogen being treated and the severity of the pathogen infestation. Thus, the dosage regimen utilizing the defensin and peptide is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the subject; the severity of the condition to be treated; the route of administration; and the particular defensin thereof employed. A physician, clinician or veterinarian of ordinary skill can readily determine and, if necessary, prescribe the effective amount of the defensin required to prevent, counter or arrest the progress of pathogen infestation. Slow release formulations are also contemplated herein.

[0075] Dragee cores are provided with suitable coatings. For this purpose, concentrated

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sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[0076] Defensin-agent preparations include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

[0077] In another embodiment, microorganisms belonging to genera or species of normal flora are genetically engineered to produce a protein such as a defensin and/or a proteinase inhibitor. Such microorganisms may be used as a probiotic to colonize gut regions or skin surface regions which may be used in conjunction with an anti-fungal chemical agent. Alternatively, the defensin and/or proteinase inhibitor is provided as a cell extract.

[0078] Another aspect provides a protocol or method for treating or preventing an animal including a mammalian such as a human subject from being infected or infested with a pathogen, the protocol or method comprising applying to the subject an anti-pathogen effective amount of a composition comprising the plant defensin and either a proteinase inhibitor or a chemical agent having anti-pathogen properties as described herein.

[0079] The term "applying" includes contacting and exposing.

[0080] The present defensin and peptide may be manufactured based on its amino acid sequence using standard stepwise addition of one or more amino acid residues using, for example, a peptide or protein synthesizer. Alternatively, the defensin and peptide may be made by recombinant means or is purified from a natural source or is in an extract from a

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plant or other biological source. For example, extracts may be used in a body wash or shampoo.

[0081] As indicated above, the combination of the defensin and other agent exhibits improved or enhanced anti-pathogen activity.

[0082] Still another aspect provides a method for reducing or controlling pathogen infestation on or in a human or animal subject the method comprising topically applying a combination of a plant defensin and either a proteinase inhibitor or chemical agent having anti-pathogen properties to a potentially infected surface region on the human or animal. Hence, animal and in particular mammalian such as human anti-pathogen medicaments are contemplated herein. In an embodiment, the medicament is in the form of a powder, spray, atomizer, nanoparticle, gel, paste, impregnated bandage, paint, aerosol, drench or other liquid. The anti-pathogen formulation may also be a slow release composition. The formulation may be used to treat an infected subject or as a preventative. In an example, the formulation is used to prevent or treat fungal infection or infestation of nails, scalp, eyes, ears, nose sinuses, hooves, reproductive tissues, lungs, and skin areas such as between toes.

[0083] As used herein, "comprising" is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, "consisting of" excludes any element, step, or ingredient not specified in the claim element. As used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim. Any recitation herein of the term "comprising", particularly in a description of components of a composition or in a description of elements of a device, is understood to encompass those compositions and methods consisting essentially of and consisting of the recited components or elements. The present disclosure illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein.

[0084] When a Markush group or other grouping is used herein, all individual members of

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the group and all combinations and subcombinations possible of the group are intended to be individually included in the disclosure.

[0085] When a range is recited herein, it is intended that all subranges within the stated range, and all integer values within the stated range, are intended, as if each subrange and integer value was recited.

[0086] Once a permeabilizing defensin is identified together with the concentration range providing optimal permeabilization, it is tested with different combinations of peptides against selected pathogens.

EXAMPLES

[0087] Aspects disclosed and enabled herein are now described in the following non-limiting Examples.

Methods

*Purification of defensins from *Pichia pastoris**

[0088] A single pPINK-defensin *P. pastoris* PichiaPink (Trademark) strain 1 colony is used to inoculate 25 mL of BMG medium (described in the Invitrogen *Pichia* Expression Manual) in a 250 mL flask and that is incubated over for 2-3 days in a 30°C shaking incubator (140 rpm). The culture is used to inoculate 200 mL of BMG in a 1 L baffled flask which is placed in a 30°C shaking incubator (140 rpm) overnight. The cells are harvested by centrifugation (2,500 x g, 10 min, 4°C) and resuspended into 1 L of BMM medium in a 5 L baffled flask and incubated in a 28°C shaking incubator for 3 days. The cultures are induced at t=24 and 48h. The expression medium is separated from cells by centrifugation (6000 rpm, 20 minutes). The medium is adjusted to pH 3.0 before it is applied to an SP Sepharose column (1 cm x 1 cm, Amersham Biosciences) pre-equilibrated with 100mM potassium phosphate buffer, pH 6.0. The column is then washed with 100 mL of 100mM potassium phosphate buffer, pH 6.0 and bound protein is eluted in 10x10 mL of 100mM potassium phosphate buffer containing 500 mM NaCl. Eluted proteins are concentrated down to 1 mL using a centrifugal column and washed 5x using sterile milli Q ultrapure water. The protein concentration of Pichia-expressed defensin is determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co.) with bovine serum albumin (BSA) as the protein standard.

Preparation of proteinase inhibitors

[0089] Total RNA was extracted from tomato (*Solanum lycopersicum*) leaf using the RNeasy Plant Mini Kit (Qiagen). cDNA was then synthesized using SuperScript II Reverse Transcriptase (Life Technologies). DNA encoding the mature form of SlCys9 (Genbank

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accession number AF198388) without the C-terminal extension (previously designated tSlCys9 (Girard *et al.* (2007) *New Phytol* 173:841-851) but here referred to as SlCys9N) was amplified and inserted into the pHUE expression vector (Cantanzariti *et al.* (2004) *Protein Science* 13:1331-1339) using Sac II and Sac I. For HvCPI6, genomic DNA was extracted from barley (*Hordeum vulgare*) leaf using the Dneasy Plant Mini Kit (Qiagen). DNA encoding the mature form of HvCPI6 (Genbank accession number AJ748341) was amplified and inserted into the pHUE expression vector (Cantanzariti *et al.* (2004) *supra*) using Sac II and Sac I. Plasmid DNA was isolated and then used to transform *E. coli* Tuner (DE3) pLysS (Novagen) cells.

[0090] Single colonies of *E. coli* Tuner (DE3) pLysS (Novagen) were used to inoculate 2YT media (10 mL, 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing ampicillin (0.1 mg/mL), chloramphenicol (0.34 mg/mL), tetracycline (0.1 mg/mL) and kanamycin (0.015 mg/mL) and grown overnight with shaking at 37°C. This culture was used to inoculate 2YT media (500 mL) containing ampicillin (0.1 mg/mL), chloramphenicol (0.34 mg/mL), tetracycline (0.1 mg/mL) and kanamycin (0.015 mg/mL) which was then grown for 4 hours to an optical density (600 nm) of ~ 1.0. IPTG was then added (0.5 mM final concentration) and the culture grown for a further 4 hours at 37°C. Cells were harvested by centrifugation (4,000 g at 4°C for 20 minutes), resuspended in native lysis buffer (20 mL per litre cell culture, 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and frozen at -80°C. Cells were then thawed and treated with lysozyme (5 mg per 25 mL resuspended cells) for 20 minutes at 4°C. DNase I (125 uL, 2 mg/mL in 20 % glycerol, 75 mM NaCl) and MgCl₂ (125 uL, 1 M) were then added and the samples incubated at room temperature for 40 minutes on a rocking platform. The samples were then sonicated for 2 x 30 s on ice (80 % power, Branson sonifier 450) and centrifuged (20,000 g at 4°C for 30 minutes). The hexahistidine-tagged ubiquitin-fusion proteins (His6-Ub- SlCys9N/HVCP16) were then purified from the protein extracts by immobilized metal affinity chromatography (IMAC) under native conditions using Ni-NTA resin (1.5 mL to ~ 25 mL native protein extract, Qiagen) according to the manufacturer's instructions. Recombinant proteins were eluted using elution buffer (250 mM imidazole, 200 mM NaCl, 50 mM NaH₂PO₄, pH 8.0). The imidazole was removed by applying the eluted protein to a prepacked Sephadex G50 gel filtration column (PD-10, Amersham)

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equilibrated with 50 mM Tris.Cl, 100 mM NaCl, pH 8.0.

[0091] The hexahistidine-tagged ubiquitin was cleaved from the recombinant proteins using the deubiquitylating enzyme 6H.Usp2-cc (Catanzariti *et al.* (2004) *Protein Sci* 13:1331-1339). The cleaved tag was removed by another round of IMAC with the deubiquitylated protease inhibitors as the unbound protein. This was then further purified by reversed-phase HPLC.

[0092] Recombinant HvCPI6 and SlCys9N were prepared as 10 times stock solutions in H₂O. Bovine pancreatic trypsin inhibitor (BPTI) was purchased from Sigma (product number T0256) and diluted to a 10 times stock solution in H₂O.

Preparation of chemical fungicides

[0093] Chemical fungicides were sourced commercially and were prepared as 10x stock solutions in H₂O (caspofungin and fenpropimorph) or as a 100x stock in methanol (terbinafine).

Preparation of spores

[0094] Spores are isolated from sporulating fungus spp. growing on yeast extract peptone dextrose agar (*Candida albicans*, *Cryptococcus gattii* – 1.5×10^6) or ½ strength Sabouraud dextrose agar (*Trichophyton interdigitale*, *Microsporium fulvum* – 5×10^4). Spores are removed from the plates by the addition of ½ strength potato dextrose broth (PDB). Spore concentrations are measured using a haemocytometer.

Antifungal assays

[0095] Antifungal assays were conducted in 96 well microtitre plates. Wells were loaded with 10 µL of filter sterilized (0.22 µm syringe filter, Millipore) defensin (10x stock for each final concentration) or water, 10 µL of filter sterilized (0.22 µm syringe filter, Millipore) proteinase inhibitor or chemical fungicide (10x stock for each final

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concentration) or water and 80 μ L of 5×10^3 spores/mL in $\frac{1}{2}$ strength PDB. The plates were incubated at 24°C. Fungal growth was assayed by measuring optical density at 595nm (A595) using a microtitre plate reader (SpectraMax Pro M2; Molecular Devices). Growth was allowed to proceed until the optical density (OD) of the fungus in the absence of any test defensin reached an OD of 0.2. Each test was performed in duplicate.

[0096] Synergy is classified as the difference between the observed % fungal growth inhibition caused by the combination of a defensin and a proteinase inhibitor (or a defensin and a chemical fungicide) (Io value) and the expected % fungal growth inhibition of the defensin and the proteinase inhibitor (or the defensin and the chemical fungicide) based on the sum of the % fungal growth inhibition of the defensin and the proteinase inhibitor (or the defensin and the chemical fungicide) on their own (Ee value calculated according to the Limpel formula used by Richer *et al.* (1987) *supra*). The difference, Io-Ee, is the synergy value. A synergy value up to 15 means no significant synergy; 15-30 is a low level of synergy; 30-60 is a medium level of synergy; and >60 is a high level of synergy. Synergy calculations are presented in Tables 2 through 7 wherein, as indicated above, Ee is the expected effect from the additive response according to Limpel's formula expressed as percent inhibition and Io is the percent inhibition observed. Synergy occurs when Io values are higher than Ee values.

EXAMPLE 1***Permeabilizing defensin and proteinase inhibitor***

[0097] Defensins include a *Solanaceous* Class II defensin (NaD1), an artificial variant (HXP4) and Class I defensins (HXL001, HXL004, HXL008, HXL012, HXL013, HXL015) which are permeabilizing defensins. These are partnered with the proteinase inhibitors BPTI, HvCPI6 or S1Cys9N. The results are shown in Tables 2 to 5.

[0098] The results of synergistic inhibition of *Candida albicans* are shown in Table 2.

Table 2
Candida albicans

Permeabilizing defensin	Proteinase Inhibitor	Expected inhibition	Observed inhibition	Synergy
NaD1 (2 μ M)	BPTI (0.625 μ M)	29.7	94.1	64.4
	HvCPI6 (2.5 μ M)	0.0	64.7	64.7
	S1Cys9N (2.5 μ M)	31.9	94.8	62.9
HXP4 (2 μ M)	BPTI (0.625 μ M)	16.2	100	83.8
	HvCPI6 (2.5 μ M)	15.9	93.5	77.6
	S1Cys9N (2.5 μ M)	33	92.4	59.4
HXL001 (4 μ M)	BPTI (0.625 μ M)	2.6	72.6	70.1
	HvCPI6 (2.5 μ M)	59.2	84.2	25.0
	S1Cys9N (2 μ M)	68.1	85.0	16.9
HXL004 (2 μ M)	BPTI (0.625 μ M)	9.6	91.2	81.6
	HvCPI6 (2.5 μ M)	63.6	93.5	29.9
	S1Cys9N (2.5 μ M)	55.2	81.8	26.6
HXL008 (2 μ M)	BPTI (0.625 μ M)	0.0	100.0	100.0
	HvCPI6 (2.5 μ M)	14.8	63.8	49.0
	S1Cys9N (2.5 μ M)	30.2	98.9	68.7
HXL012 (4 μ M)	BPTI (0.625 μ M)	14.8	89.7	74.9
	HvCPI6 (2.5 μ M)	12.3	100.0	87.7
	S1Cys9N (2.5 μ M)	34.5	97.3	62.8
HXL013 (1 μ M)	HvCPI6 (2.5 μ M)	10.1	100.0	89.9
	S1Cys9N (2.5 μ M)	13.8	100.0	86.2
HXL015 (1 μ M)	BPTI (0.625 μ M)	28.1	100.0	71.9
	HvCPI6 (2.5 μ M)	0.0	100.0	100.0
	S1Cys9N (2.5 μ M)	0.0	94.7	94.7

[0099] The results of synergistic inhibition of *Cryptococcus gattii* are shown in Table 3.

Table 3
Cryptococcus gattii

Permeabilizing defensin	Proteinase Inhibitor	Expected inhibition	Observed inhibition	Synergy
NaD1 (2 μ M)	BPTI (0.625 μ M)	22.0	95.6	73.7
	HvCPI6 (2.5 μ M)	32.1	96.2	64.1
	SlCys9N (2.5 μ M)	0.0	98.8	98.8
HXP4 (0.5 μ M)	BPTI (0.625 μ M)	48.8	97.4	48.6
	HvCPI6 (2.5 μ M)	18.6	97.4	78.8
	SlCys9N (2.5 μ M)	0.0	99.3	99.3

[0100] The results of synergistic inhibition of *Trichophyton interdigitale* are shown in Table 4.

Table 4
Trichophyton interdigitale

Permeabilizing defensin	Proteinase Inhibitor	Expected inhibition	Observed inhibition	Synergy
NaD1 (1 μ M)	BPTI (0.625 μ M)	57.9	89.1	31.2
	HvCPI6 (2.5 μ M)	33.5	71.6	38.1
	SlCys9N (2.5 μ M)	28.1	81.1	53.0
HXP4 (0.5 μ M)	BPTI (0.625 μ M)	32.9	69.0	36.1
	HvCPI6 (2.5 μ M)	31.1	56.8	25.7
	SlCys9N (2.5 μ M)	9.7	53.0	43.4
HXL001 (0.5 μ M)	BPTI (0.625 μ M)	36.9	67.4	30.5
	HvCPI6 (2.5 μ M)	32.5	84.7	52.2
	SlCys9N (2.5 μ M)	16.8	70.2	53.4

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[0101] The results of synergistic inhibition of *Microsporum fulvum* are shown in Table 5.

Table 5*Microsporum fulvum*

Permeabilizing defensin	Proteinase Inhibitor	Expected inhibition	Observed inhibition	Synergy
NaD1 (0.5 μ M)	BPTI (0.625 μ M)	34.3	52.9	18.6
	HvCPI6 (0.625 μ M)	19.0	43.8	24.8
	SlCys9N (5 μ M)	40.7	79.9	39.2

EXAMPLE 2***Permeabilizing defensin and fungicide***

[0102] Defensins include a *Solanaceous* Class II defensin (NaD1), an artificial variant (HXP4) and Class I defensins (HXL001, HXL002, HXL004, HXL008, HXL009, HXL012, HXL013, HXL015) which are permeabilizing defensins. These are partnered with the chemical fungicides caspofungin, fenpropimorph and terbinafine.

[0103] The results for synergistic inhibition of *Candida albicans* is shown in Table 6.

Table 6
Candida albicans

Fungicide	Permeabilizing defensin	Expected inhibition	Observed inhibition	Synergy
Caspofungin (2 ng/ml)	NaD1 (1 μ M)	1.2	61.7	60.5
	HXL004(1 μ M)	14.0	92.3	78.3
	HXL013 (1 μ M)	4.3	90.8	86.5
	HXP4 (1.5 μ M)	14.5	64.5	50.0
Caspofungin (4 ng/ml)	HXL001 (2.5 μ M)	11.7	73.4	61.7
	HXL002 (2.5 μ M)	6.1	40.8	34.7
	HXL009 (2.5 μ M)	3.7	65.2	61.5
	HXL012 (2.5 μ M)	0	51.2	51.2
Fenpropimorph (2 ug/ml)	HXP4 (1.5 μ M)	15	97.6	82.6
	HXL008 (2 μ M)	46.7	87.3	40.6
	HXL015 (1 μ M)	19	71.8	52.8
Terbinafine (4 ng/ml)	HXP4 (1.5 μ M)	44.8	82.7	37.9

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EXAMPLE 3*Non-permeabilizing defensin and fungicide*

[0104] Table 7 shows the no to low synergy when a non-permeabilizing defensin is used.

Table 7
Candida albicans

Fungicide	Non-permeabilizing defensin	Expected inhibition	Observed inhibition	Synergy
Caspofungin (2 ng/ml)	DmAMP1 (1 μ M)	18.5	30.0	11.5
	RsAFP2(1 μ M)	1.7	19.0	17.3

[0105] Those skilled in the art will appreciate that the disclosure described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure contemplates all such variations and modifications. The disclosure also enables all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features or compositions or compounds.

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CLAIMS:

1. A method for inhibiting growth or infestation of a fungal pathogen in or on a human or animal subject, said method comprising contacting the fungal pathogen with an effective amount of a combined synergistic amount of a plant permeabilizing defensin or a functional natural or synthetic derivative or variant thereof and a chemical pathogenicidic or pathogenostatic agent effective to inhibit growth or infestation of the pathogen in or on the human or animal subject, wherein the chemical agent is selected from the list consisting of terbinafine, fenpropimorph and a beta-glucan synthase inhibitor, and wherein the fungal pathogen is a *Candida spp.*
2. The method of Claim 1 wherein the plant permeabilizing defensin is selected from the list consisting of NaD1, TPP3, PhD1, PhD1A, PhD2, FST, NoD173, HXL001, HXL002, HXL004, HXL007, HXL008, HXL009, HXL012, HXL013, HXL015, HXL035 and HXL036.
3. The method of Claim 1 or Claim 2 wherein the plant permeabilizing defensin is a *Solanaceous* Class II defensin or a functional natural or synthetic derivative or variant thereof.
4. The method of Claim 1 or Claim 2 wherein a defensin variant comprises a Loop1B from a Class I defensin replacing the corresponding Loop1B from a *Solanaceous* Class II defensin.
5. The method of Claim 4 wherein the defensin variant is selected from the list consisting of HXP4, HXP34, HXP35, HXP37, HXP58, HXP72, HXP91, HXP92, HXP95 and HXP107.
6. A method for inhibiting growth or infestation of a fungal pathogen, the method comprising contacting the fungal pathogen with a combined synergistic amount of a plant defensin selected from the list consisting of NaD1, TPP3, PhD1, PhD1A, PhD2, FST, NoD173, HXL001, HXL002, HXL004, HXL007, HXL008, HXL009, HXL012, HXL013, HXL015, HXL035 and HXL036 or a variant or derivative of a defensin selected from the list consisting of HXP4, HXP34, HXP35, HXP37, HXP58, HXP72, HXP91, HXP92, HXP95 and

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HXP107 and a chemical agent selected from the list consisting of terbinafine, fenpropimorph and a beta-glucan synthase inhibitor to facilitate inhibition or growth of the fungal pathogen in or on the human or animal subject, and wherein the fungal pathogen is a *Candida spp.*

7. The method of any one of Claims 1 to 6, wherein the beta-glucan synthase inhibitor is an echinocandin.
8. The method of Claim 7 wherein the echinocandin is caspofungin.
9. The method of Claim 6, wherein the fungal pathogen is selected from the group consisting of *Candida albicans*, *Candida dubliniensis*, *Candida famata*, *Candida glabrata*, *Candida guilliermondii*, *Candida haemulonii*, *Candida kefyr*, *Candida krusei*, *Candida lusitanae*, *Candida norvegensis*, *Candida parapsilosis*, *Candida tropicalis* and *Candida viswanathii*.
10. The method of Claim 9, wherein the fungal pathogen is *Candida albicans*.
11. The method of Claim 9, wherein the fungal pathogen is *Candida glabrata*.
12. The method of any one of Claims 1 to 11, wherein the defensin and the chemical agent are topically applied to a human or animal.
13. The method of any one of Claims 1 to 13, wherein the defensin is produced by a genetically modified microorganism or is provided by an extract of a plant or cell.