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Peptide-based Antifungal Therapies against Emerging Infections

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Abstract

Acquired drug resistance to mycotic infections is rapidly emerging as a major medical problem. Opportunistic fungal infections create therapeutic challenges, particularly in high risk immunocompromised patients with AIDS, cancer, and those undergoing transplantation. Higher mortality and/or morbidity rates due to invasive mycosis have been increasing over the last 20 years, and in light of growing resistance to commonly used antibiotics, novel antifungal drugs and approaches are required. Currently there is considerable interest in antifungal peptides that are ubiquitous in plant and animal kingdoms. These small cationic peptides may have specific targets or may be multifunctional in their mechanism of action. On the basis of recent advances in protein engineering and solid phase syntheses, the utility and potential of selected peptides as efficient antifungal drugs with acceptable toxicity profiles are being realized. This review will discuss recent advances in peptide therapy for opportunistic fungal infections.

INTRODUCTION

In an era of increased incidence of fungal infections in immunocompromised patients (1,2) and greater resistance to “frontline” antifungal therapies (3), there is a growing need to discover new antifungal therapies. Although newer azole derivatives such as voriconazole are more effective and have cidal activity against filamentous fungi such *Aspergillus fumigatus* (4), these derivatives are fungistatic and not fungicidal against pathogenic yeasts; the inability to kill yeasts leads to resistance to azole in prolonged infections and increases the likelihood that these agents will lack efficacy in severe *Candida* infections in immunosuppressed patients. Amphotericin B has also been commonly used to treat serious fungal infections, but in contrast to azoles, amphotericin B is fungicidal against yeasts. Nevertheless, resistance to amphotericin B is slowly developing in selected *Candida* species (5) and there are significant side effects associated with its use, including nephrotoxicity. Although recent antifungal agents, including the peptide-based agents, micafungin and caspofungin, have been developed and are very promising, resistance to these therapies has already been reported (6–8) and will no doubt become more widespread. The development of resistance to current antifungal agents, the limited efficacy, and the side effects associated with several of these agents increase the importance of continued development of new alternative approaches. This review will examine both synthetic and natural peptides as antifungal therapies, and in this context, we will divide this review into peptides that have a primarily antifungal mechanism of action and peptides that broadly inhibit microbes including bacteria, fungi, and enveloped viruses (9). Because

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there are a large and diverse number of antifungal peptides in nature, we will mainly focus on those that show promise in treating agricultural and human diseases.

Overview of Antimicrobial and Antifungal Peptides

Antimicrobial and antimycotic peptides are small cationic and amphipathic molecules, generally with fewer than 50 amino acids. These peptides are omnipresent and have been isolated from prokaryotes and eukaryotes in the plant, bacterial, fungal, and animal kingdoms (10–13). Nature has strategically placed antimicrobial and antifungal peptides as first line of defenses between the host organism and its surrounding environment, because these peptides are able to inhibit quickly a wide spectrum of infectious microbes without significant toxicity to the host organism. When insects are infected within a short period of time, they secrete an array of cationic peptides to combat the invading organism (14). Although antimicrobial peptides (AMP) are the primary means of combating organisms in lower forms of life, these peptides have an adjunct role to the immune system in phylogenetically more advanced organisms. Indeed, cationic peptides in humans have an important role and they are produced and secreted by several different tissues, including salivary glands, skin, eye, liver, as well as epithelial and platelet cells and neutrophils (15). Several antifungal peptides display selective toxicity for the microbial target by identifying conserved molecular determinants of pathogens (16,17). A classic example is the echinocandin family which targets 1,3 β glucan synthase, an enzyme essential for cell wall integrity of fungi (18). In most instances, however, AMP are less specific in their targeting and this results in their exhibiting a broad spectrum of inhibitory/cidal activity not only against fungi but also against bacteria and envelope-containing viruses (19). Broad spectrum AMP often target and lyse the membrane of the microbe, yet these peptides frequently have less proclivity to lyse mammalian cell membranes such as those of red blood cells. The interaction between AMP and target microbes is complex, but the positive charge of the peptides is essential to its binding with negatively charged membrane/wall elements such as the mannoproteins in yeasts (19). Moreover, despite targeting and lysing microbial membranes, the potencies and spectra of activities of these broad spectrum AMPs against different classes of microbes vary and depend on the membrane composition of the pathogen and the structure of the peptide. Much remains to be learned about the subtle differences in microbial membranes that may affect efficacy of the AMP.

Although the focus of this review is on the direct action of peptides against disease-causing fungi, accumulating evidence suggests that the antifungal activity of AMPs is multifactorial. For example, AMPs stimulate the immune system in mammals by several mechanisms: 1) activation of T-cells; 2) stimulation of Toll-like receptors; 3) amplifying phagocyte action; 4) activation of dendritic cells; and 5) chemo-attraction of neutrophils (20–25). Moreover, these activated cells and receptors may reduce the growth of fungi *in vivo* by modifying levels of various cytokines, chemokines, and integrins (26,27). Thus, similar to other antifungal agents (28,29), the interplay between antifungal peptides, their modulation of the immune system, and the host immune status will likely determine the efficacy of the peptide. In addition to their role in providing immunosurveillance against pathogens and maintaining a healthy floral milieu, studies have shown the potential of antimicrobial cationic peptides in cancer and gene therapy (30–33). As peptide-engineering methods develop, the potential for producing sufficient amounts of naturally occurring peptides increases significantly. As a result, the antifungal peptides offer promise for future treatment of infectious diseases in a diverse range of organisms including humans. Figure 1 shows representatives of specific and broad-spectrum antifungal peptides as well as their mechanisms of action.

Because of the disparate structure of antifungal peptides and the incomplete knowledge of their mechanisms of action, classification of the various antifungal peptides is a daunting task. Whereas some lipopeptides (e.g., echinocandins) or histidine-rich (e.g., the linear histatins or

branched HK) peptides have primarily antifungal activity, membrane-disrupting peptides (e.g., magainins, protegrins) inhibit a diverse group of microorganisms including fungi, bacteria, and viruses. Structurally, linear cationic antifungal peptides (e.g., LL-37, magainins) form α-helical structures in a hydrophobic milieu while cysteine-containing peptides containing from one to multiple disulfide bonds (e.g., protegrins and defensins) form β-sheet enriched structures. The formation of these α–helical and/or β sheet secondary structures may increase the amphipathicity of the peptides and enable them to act specifically with their targets in the fungal membrane. In addition to histidine-rich peptides such as histatins, other peptides (e.g., apidaecins, indolicidin) have a high percentage of certain amino acids such as proline and tryptophan. Interestingly, potent antifungal linear peptide fragments from larger proteins (e.g., lactoferrin, casein, and lysozyme) are able to inhibit fungi because of multiple direct and indirect effects (34). Notably, for optimal antimicrobial activity, it may be necessary for the peptide structures to undergo post-translational modifications including glycosylation, formation of D-amino acid enantiomers, amidation, halogenation, or phosphorylation (20, 35–39).

Nearly 1200 antimicrobial peptides have now been identified. In this review, we have selected examples of peptides that have antifungal properties with varied mechanisms of action (Fig. 1). For supplementary reviews on antimicrobial peptides, see reviews by Jensen and Hancock (40), Yeaman (41), De Lucca(42), and Bulet (13))

PEPTIDES WITH PRIMARILY ANTIFUNGAL PROPERTIES

Naturally occurring peptides with primarily or exclusively antifungal properties are less ubiquitous than those with broad antimicrobial action. From an evolutionary perspective it was important for nature to form peptides that were functionally omnipotent and could protect all life forms from a great variety of infectious pathogens. Thus, the vast majority of small peptides are actually antimicrobial affecting bacteria, fungi, and enveloped viruses (43). Nevertheless there are some peptides, both natural and synthetic, which exhibit primarily antifungal activity. The potential advantage of these is that the therapeutic window for peptides specific for fungi is greater than that of peptides with broad antimicrobial activity. In contrast to broad spectrum antimicrobial peptides, which induce membrane lysis, most of these antifungal peptides have specific targets that are intracellular, on the cell membrane, or on the cell wall. Because of the diverse targets, the structure of these AMP can vary significantly and includes linear, open ended cysteine-rich cyclic peptides, and cyclic lipopeptides (see Table 1).

1,3-β-Glucan synthesis inhibitors

These specific fungal inhibitors are cyclic lipoproteins that noncompetively inhibit the multiunit membrane-integrated enzyme, β-glucan synthase, critical for cell wall integrity. Inhibition of β-glucan synthase results in destabilizing the cell wall, leading to susceptibility to osmotic stresses and cell lysis. In addition to the cell wall, 1,3- β-glucans have a role in the division septum and assembly of the acropore wall; consequently, these structures are also sensitive to the synthase inhibitors. β-Glucan synthase has a widespread distribution in fungi including *Candida*, *Aspergillus*, *Cryptococcus*, and *Pneumocystis* species (spp.). Mycelious fungi such as *Aspergillus* spp., however, are less sensitive to these inhibitors because the synthase is found primarily in tips of the growing hyphae. Inhibition of β-glucan synthase results in negative feedback, causing cell cycle arrest. The family of echinocandins consists of several lipopeptides that differ slightly in their peptide core; this family includes the echinocandins, pneumocandins, molundocandins, aculeacins, and WF11899 (44–46). Notably, members of the echinocandin family are the only peptides thus far approved for severe localized (e.g., pneumonitis) or systemic fungal infections.

Echinocandins and Pneumocandins—Several analogs from these two classes of β-glucan synthase inhibitors have shown promise in preclinical or clinical studies for treatment of invasive and systemic *Candida* and *Aspergillus* infections. Of the three subfamilies of echinocandins (B, C and D), analogs of group B have been most useful in the development of antifungal drugs. Echinocandin B, produced by *Aspergillus nidulans* and *A. rugulosus* (47), was found to have potent antifungal activity with an MIC between 0.20 and 0.35μg/ml[1] for *Candida* spp. (47). Nevertheless, it was an unsuitable antifungal candidate because it induced lysis of red blood cells. Compared to echinocandins, pneumocandins have a broader spectrum of fungicidal activities (48,49). This group of cyclic lipopeptides including the most studied, pneumocandin A_0 (L-671,329)is produced by *Zalerion asboricola* and has shown potent activity against *Candida* spp. and *Pneumocystis carinii* (50). Similar to echinocandin B, pneumocandin A_0 also caused hemolysis. After significant research and screening a number of analogs with varied N-acyl side chain substitutions, the unwanted side effect of hemolysis from these lipopeptides was mitigated with the echinocandin analog, cilofungin (LY121019). While there was no effect on its antifungal activities against *A. fumigatus* and *Candida* spp. (51), cilofungin had a 10-fold lower hemolysis than echinocandin B. Nevertheless, clinical trials on cilofungin were stopped because of side effects (52).

Analogs with improved efficacy and safety profile and greater aqueous solubility have now been developed and have received FDA approval for antifungal treatment (53). Currently, there are three approved echinocandins/pneumocandins: caspofungin (second generation pneumocandin, MK-0991) was approved in 2001, micafungin (an echinocandin, FK463) in 2004, while anidulafungin (an echinocandin, LY30366) was most recently approved in 2006. All three drugs have fungicidal activity toward the majority of *Candida* spp. (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*, and *C. krusei*) including those that are resistant to fluconazole or amphotericin; moreover, these antifungal lipopeptides are fungistatic toward Aspergillus species. As a representative for these lipopeptides, the MIC of micafungin ranged from 0.0156 to 2 μg/ml for *Candida* spp. while for *Aspergillus* spp., the MIC ranged from 0.0078 to 0.0156μg/ml (54). There are a number of fungi in which the microbial activities of these echinocandins/pneumocandins are less effective (e.g., *C. parapsilosis*, *C. guilliermondii*, *Cryptococcus neoformans*, mucormycoses (*Rhizopus oryzae*), and yeast forms of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidiodies immitis*). Currently, these three lipopeptides have been approved for various *Candida* infections and caspofungin has been approved to treat invasive aspergillosis when patients are unable to tolerate or are resistant to first-line therapies. Because there are a number of fungi in which the echinocandin family show poor activity, care must be taken to select the appropriate clinical setting to administer these drugs.

Other β-glucan synthase inhibitors

In addition to echinocandins/pneumocandins, there are a number of other related echinocandin family members that will be mentioned briefly. To date, these cyclic lipoprotein analogs have not been clinically approved, and in general, the following classes have reduced antifungal activity and/or greater toxicity compared to the pneumocandins/echinocandins discussed previously.

Aculeacins (A-D, F), isolated from *Aspergillus aculeatus*, have potent antifungal activity (55–57). In general, aculeacins have a MIC of less than 0.31 μg/ml for most *Candida* spp., but they are not active against *C. tropicalis* or most filamentous fungi (58). When aculeacin A was compared with pneumocandin A_0, the aculeacin induced significantly more hemolysis at a

[1]Because investigators use different MIC units (molar vs. weight/vol), the approximate molecular weights are listed in Tables 1,2 to enable conversion and comparison of the activities of AMP.

lower concentration and was slightly less effective *in vivo* against systemic *C. albicans* infection in a mouse model (50).

Mulundocandins differ structurally from echinocandins by one amino acid; threonine is substituted by serine, and the lipophilic side chain is 12-methylmyristoyl and not lineoyl (59). Mulundocandins are produced by Aspergillus syndowi var. mulundenis (59,60) and are effective against *C. albican*s (MIC, 0.5–4.0 μg/ml), *C. glabrata* (MIC, 2.0 to 4.0 μg/ml), and *C. tropicalis* (MIC, 1.0–8.0 μg/ml). Against other species of *Candida*, mulundocandins are less active and show little to no activity against *C. neoformans* or filamentous fungi (61).

WF11899A: WF11899A, B and C possess potent anti-Candida activities that are superior to cilofungin, and equivalent to fluconazole; unfortunately, they lyse mouse red blood cells at low concentrations (62).

Inhibitors of Chitin in the Cell Wall

Nikkomycins, produced by *Streptomyces tendae* and *S. ansochromogenes*, and ***polyoxins***, produced by *Streptomyces cacaoi*, are the most widely studied peptidyl nucleoside inhibitors of chitin synthase (63–65). Synthase inhibitors such nikkomycin are structural analogs of uridine diphosphate N-acetylglucosamine, a major constituent of chitin. Absent in vertebrates, chitin is a cell wall component that is essential to maintain the structural integrity of the fungus. Nikkomycins are able to inhibit chitin synthesis in *C. albicans* both *in vitro* and *in vivo* studies (66) and they are not toxic to human cells; they also show significant activity against *C. immitis*, *B. dermatides*, and moderate activity against *H. capsulatum* (67,68), but these agents are not active against filamentous fungi. Despite their advantages, the use of these inhibitors has been limited because of unfavorable pharmacokinetics.

Aureobasidins: There are 18 members of the Aureobasidins family, produced by *Aureobasidium pullulans.* They are cyclic depsipeptide lipophilic antibiotics made up of eight amino acids and an α-hydroxyacid (69). Two modes of action have been proposed for aureobasidins: one is based on disruption of cell wall/membranes by altering the assembly of actin and chitin (70) and the other is based on interrupted synthesis of sphingolipids (71). Several members of the Aureobasidin family are active against *Candida* spp. (e.g., MIC, Aureobasidin A, <0.05 to 0.2 μg/ml), and *C. neoformans* (e.g., MIC, Aureobasidin A, 0.78 μg/ml) (72).

Membrane-active Selective AMPs

Rs-ARF2, isolated from radish seeds, is a 50 amino-acid residue plant defensin that has an α-helix and three-stranded β-sheets stabilized by four disulfide bridges. Rs-ARF2 shares structural and functional homology with other plant defensins, **HsAFP1** and **DmAMP1**, and the insect defensin, **heliomicin** (73–75). Notably, these defensins have no significant effect on bacteria, but have marked antifungal effects. Rs-ARF2 causes rapid K^+ efflux, Ca^{2+} uptake, and alkalinization of the medium by targeting the fungus-specific membrane glucosylceramide and by inducing membrane permeability. After targeting the ceramide in the membrane, this defensin also induces reactive oxygen species intracellularly that are toxic to the fungi. Following 7.5 h of incubation, RsAFP2 (10 μM) inhibited *C. albicans* and *C. krusei* by 99.8% and 91.1%, respectively (73). *C. glabrata*, which does not contain this fungus-specific ceramide, was not inhibited. Furthermore, the MICs of RsAFP2 toward *A. flavus* and *Fusarium solani* were 0.7 and 0.04 μM, respectively. Analogs of Rs-ARF2, in which arginines replaced neutral amino acids, were more effective against *F. solani*. Moreover, replacement of cysteines with alpha-aminobutyric acid improved the antifungal potency of Rs-ARF2 (76). Unlike iturins discussed later in this section, Rs-ARF2 and its close analogs have little cytotoxicity to mammalian cells at dosages that are inhibitory to fungal pathogens.

Drosomycin, an insect defensin structurally related to Rs-ARF2, is an inducible 44-residue cysteine-rich peptide produced by *Drosophila melanogaster* (74,75). Despite its sequence homology with Rs-ARF2, the underlying antifungal mechanism of drosomycin remains unclear. Based on the interaction of drosomycin with the voltage-gated sodium channel in *D. melanogaster*, Cohen and colleagues have suggested that this interaction may have a role in the inhibition of microbial pathogens (77). More is understood about the signal transduction pathways that up-regulate drosomycin levels in the hemolymph of *D. melanogaster*. After insects are challenged by bacteria, drosomycin, under the control of activated Toll receptors, reaches its peak concentration in the hemolymph at 16 h (74,78); despite its induction by bacteria, this defensin has no significant antibacterial effect (74). Drosomycin has significant fungicidal activity against filamentous fungi (e.g., MIC<5 μM, *F. oxysporum, Neurospora crassa)* (79) and recent studies have revealed its antiyeast (MIC, 12 μM, *S. cerevisiae*) activities (80).

Iturins, produced by *Bacillus subtilis*, are cyclic peptides with a lipid-soluble β-amino acid linked to an array of D and L amino acids. Iturins act on microbial membranes causing pore formation and leakage of key ions of fungi (81), but their antimicrobial activity is limited primarily to fungi, with little effect on bacteria. Unfortunately, iturins are toxic to mammalian cell membranes. In contrast to most other antimicrobial peptides which are cationic, iturins may be anionic (bafilomycin L) or neutral (iturin A). One member of the family, bafilomycin F effectively inhibits *Aspergillus niger (*MIC, 40 *μg/ml), C. Albicans (*MIC, 40 *μg/ml), C. tropicalis (*MIC, 40 *μg/ml),* and several phytopathogens such as *Mycosphaerella pinodes* (MIC, 10 μg/ml) (82). In contrast to its potent antifungal activity, bafilomycin F modestly inhibits the bacterium, *Micrococus luteus* (MIC, 200 μg/ml), but has no inhibitory effect on other bacteria tested (e.g., MIC> 400 μg/ml: *Escherichia coli* K12, *Streptomyces albus* G, *Staphylococcus aureus*). In addition, with a radial diffusion assay, Klich et al. found that Iturin A had marked antifungal activity against phytopathogens and human pathogens such as *A. flavus* (83); most fungi were inhibited at 7.7 μg/ml with no change in the zone of inhibition for several weeks. Although this group of peptides is effective against dermatomycoses in humans and animals, they induce unacceptable levels of red blood cell lysis (84). Nevertheless, there is a growing interest in substituting toxic pesticides with these peptides for treatment of fungal infections in plants.

Histidine-rich Peptides

Histatins are a group of linear cationic peptides that are isolated from human saliva and have potent and specific biological activity against fungi; their weak amphipathic character, lack of disulfide bonds, and high content of histidines distinguishes them from other cationic peptides (85). Whereas histatins form random polymers in aqueous environments, they adopt α-helical structures in hydrophobic environments and this has been posited to have a role in their antifungal activity (86,87). A key feature of histatins is their strong candidacidal activity, which includes *C. albicans, C. glabrata, C. guillermondii, C. krusei, C. lambica, C. parapsilosis, C. pseudotropicalis, C. stellatoidea,* and *C. tropicalis* (88,89), *C. neoformans* and *A. fumigatus* (90,91). This family of 12 small (3–4 kD) histidine-rich peptides is created by proteolytic cleavage of histatins-1 and -3 (92) and are exclusively found in saliva of primates (93–95). Of these peptides, histatin 5 has the strongest fungicidal activity with an MIC of 100 μM against *C. albicans* (96). P-113, a 12-mer amino acid fragment of histatin 5, is the smallest peptide that retains full anticandidal activity compared to its parent peptide (97). In addition to inhibiting yeast cells, histatin 5 and two synthetic variants (Dhar4 and 5) markedly inhibited formation of the drug-resistant biofilm of *C. albicans* (98–100). Besides mammals, arthropods also produce histidine-rich peptides that are active against certain fungi such as *C. albicans* but are not active against bacteria (101–103). Compared to histatin 5, synthetic branched histidine-rich peptides are significantly more inhibitory (about 15-fold on molar basis) against

C. albicans (104). Moreover, these branched antifungal histidine-rich peptides are highly selective, and have little toxicity toward mammalian cells.

Of interest is the rationale for use of these histidine-rich peptides that provides selective fungicidal activity. Although there are several mechanisms proposed including one study that indicates that histatins interact with an energized membrane (105), most data indicate that histidine-rich peptides must traverse the membrane and interact with an intracellular target (106–108) (see Fig. 1). Interestingly, histidine-rich peptides by our laboratory have been developed as nucleic acid carriers because of their ability to traverse membranes and disrupt acidic endosomes (32,33,109). Although we think that the mechanisms of killing fungi by histatins and branched HK polymers are closely linked, this supposition has not yet been proven.

ANTIFUNGAL PEPTIDES WITH WIDE SPECTRUM OF ANTIMICROBIAL ACTIVITY

In contrast to the peptides that specifically target fungi, most antimicrobial peptides affect a number of organisms including bacteria, fungi, and envelope-containing viruses. A common theme with most of these wide-spectrum AMP is that they lyse the membranes of the pathogen. Despite this non-specific mechanism, many of these peptides do not lyse mammalian membranes at concentrations of peptides that can inhibit the pathogen. Because the number of AMP that can affect bacteria, fungi, and viruses is extensive, we will discuss selected examples of linear and cyclic antifungal peptides made by several different species (see Table 2).

Linear peptides

Small linear primarily α-helical peptides are the most common and well-studied group of antimicrobial peptides and include families such as cecropins (110), magainins (111), and dermaseptins (112). Because α-helical amphipathic peptides differ in amino acid composition as well as in length and positive charge, their antimicrobial activity is likely determined by their global structural components rather than by the specific amino acid sequence (113). The final common pathway for these α-helical amphipathic peptides is primarily disruption of the cell membrane; consequently, these peptides have widespread activity against bacteria, fungi, and membrane-enveloped viruses. Nevertheless, accumulating data suggest that at least some of these peptides also have unique intracellular targets (114–116). In addition, many of them are able to lyse cancer cells at concentrations up to 10-fold lower than those required to lyse normal human cells (117).

Cecropins and cecropin-like peptides have a broad spectrum of antimicrobial activity (bacteria and fungi) and have primarily been isolated from the hemolymph of silkworm moths (118, 119). These peptides range from 29 to 42 amino acids in length and form α-helices in hydrophobic environments such as the plasma membrane. With few exceptions among the insect cecropins, there are two distinguishing characteristics: 1) a tryptophan in the first or second positions; and 2) an amidated C-terminal amino acid (13). Although the primary target of cecropins is the plasma membrane, cecropin A at its microbicidal dose does not affect mammalian cells and numerous studies have shown that this peptide can be administered safely to animals (120–122). At concentrations between 25 and 100 μg/ml cecropin A effectively killed *Aspergillus* spp., and at concentrations of 12.5 μg/ml the peptide effectively killed *Fusarium moniliforme* and *F. oxysporum* (123,124). Notably, genetically modified rice that expressed cecropin A gene was completely protected from the blast fungus, *Magnaporthe grisea* (125). In addition, no toxicity was reported in transgenic mice expressing a cecropin-like peptide (e.g., Shiva-1) (122). Thus, cecropins are a promising class of antifungal peptides because of their safe therapeutic profile.

Magainins, isolated from the skin of *Xenopus laevis* (the African frog), are a family of cationic amphipathic peptides that range between 21 and 26 amino acids in length and are enriched in glycine and serine residues (111,126,127). The magainin family of peptides includes magainin I, II, 2, PGLa peptides, xenopsin and the caerulein precursor fragment (128–130). Some of these family members including magainin 2 and PGLa may form heterodimers, which increases their ability to permeabilize the membranes of pathogens (131). In addition to cidal activity of magainins against gram-negative and gram-positive bacteria and protozoa, magainins have antifungal activity against *Candida* spp., *C. neoformans, and Saccharomyces cerevisiae.* Magainin 2 (Mag 2) is particularly active against *C. neoformans* (MIC, 6.25 μg/ml) with greater activity than three other cationic peptides (132). Although Mag 2 has moderate activity toward *C. albicans* (MIC > 80 μg/ml), it potently inhibits *C. glabrata* (MIC, 25.0μg/ml), *C. tropicalis* (MIC, 12.5 μg/ml), and *C. krusei* (MIC, 12.5–25.0μg/ml) (111,132). Similar to many other antimicrobial peptides, magainins show selectivity in their cidal activity toward pathogenic fungi (and bacteria). Nevertheless, the therapeutic window for natural magainins is not great enough to allow treatment of systemic infections in humans. As a result, magainin analogs or hybrid peptides with greater activity are being developed (133–135). For example, Avrahami and colleagues showed that conjugating a lipophile with magainins greatly increased their activity against *C. neoformans* (136). In addition to its membrane-lysis mechanism, Mag 2 has an alternative antifungal action by interfering with the DNA integrity of fungi (114); this study raises the question as to whether many cationic peptides kill fungi by mechanisms other than targeting the membrane.

Bombinin-H and bombinin-like peptides isolated from skin of *Bombina genus* (137,138) are glycine-rich, weakly cationic peptides with their C-terminal amino acid amidated (139). In addition to its inhibiting bacteria, bombinin-like peptides (BLP-1, 3) are active against fungi, especially *C. albicans* (MIC, BLP-1, 3–0.4 μM). Importantly, these peptides have little hemolytic activity (<10%, 15 μM). Bombinin-H peptides have varied antimicrobial and hemolytic activity (139,140). Bombinins H2 and H4, which damage cell membranes of microbes, were found to be active against *C. albicans* (MIC, H2, 3.1μM; H4, 1.6 μM) *C. guillermondii* (MIC, H2, 1.3 μM; H4, 0.7 μM), and *C. tropicalis* (MIC, H2, 1.1μM; H4, 0.6 μM) (141,142). While bombinin-H2 induces 11% hemolysis, H4 induces 28 % hemolysis at 15 μM. Other bombinin H peptides, H6 and H7, with greater hydrophobicity, have lower antimicrobial activity and induce greater hemolysis than do H2 and H4. Notably, H2 and H4 peptides are active against the spores of the fungus, *Phytophthora nicotianae*, with a minimal fungistatic concentration of 10 and 18μM (143). Although some bombinin H peptides differ in their conformations as a result of stereochemistry modifications at the second position, these bombinins have similar antimicrobial activity (144).

Dermaseptins **(S and B)** have between 28 and 34 amino acids and are lysine-rich peptides with a tryptophan characteristically at position 3. They were identified in the skin of tree frogs of the genus *Phyllomedusa* (112). By interfering with lipid layers, which leads to osmotic imbalance, dermaseptins lyse a wide spectrum of microorganisms. Remarkably, dermaseptins often exhibit synergy with one another, resulting in 100-fold increase in antimicrobial action compared to dermaseptins separately (145). In addition to their anti-bacterial, antiviral, and anti-protozoa activity, they are cidal to pathogenic fungi (146,147) including yeasts and some filamentous fungi (*A. fumigatus*) (148). For example, a synthetic dermaseptin s1 analog, a 16-mer peptide, shows marked activity against *C. albicans* (MIC, 5.8 μM), and notably this analog has little hemolytic activity (149). Interestingly, the presence of a phenyl group in position 3 selectively inhibits *C. albicans* more effectively than it inhibits bacteria. In addition to pathogenic fungi, a synthetic derivative of dermaseptin (e.g., MsrA2 (N-Met-dermaseptin B1), elicits strong antimicrobial activities against virulent phytopathogenic fungi and protects transgenic potatoes from a broad spectrum of fungal infections (150).

Metchnikowin is a 26 residue proline rich inducible peptide isolated from *Drosophila*. This peptide exhibits activity against gram-negative bacteria and filamentous fungi, but does not inhibit gram-positive bacteria. Studies with metchnikowin against fungi have been quite limited, but it has potent activity against *N. crassa* (0.5 to 1 μM).

LL-37 (CAMP18) is the only member of the cathelicidin family of host defense peptides expressed in humans (151). Members of the cathelicidin family are characterized by a conserved region and a highly variable domain that contains the mature antimicrobial peptide. In some species (cattle, pigs, goats), there are multiple antimicrobial cathelicidin peptides that show dramatic diversity in size, charge, and structure, including α-helical or β-sheet formations. LL-37, however, is an α-helical antimicrobial peptide named for the two N-terminal starting leucines and its length of 37 amino acids; the peptide is widely distributed in humans in skin, gastrointestinal tract, urinary tract and respiratory tract (152,153). The peptide is expressed by a number of cells including monocytes, neutrophils, NK cells, B and T cells (154–156) and is upregulated by immune stimuli (153) and by Vitamin D (157,158). Both LL-37 and a close analog, mCRAMP in mice, have a similar MIC range for *C. albicans* of 15- to 20 μM (159). In one study, mCRAMP was induced by *C. albicans* at the skin surface in a mouse model, demonstrating that these peptides provide a natural barrier to fungal infections. Other cathelicidin α-helical peptides have shown activity (BMAP-27, 28 from cows) against *Candida* spp. and *C. neoformans* but these were less active against filamentous fungi (160). Besides its antifungal activity, LL-37 has potent antimicrobial activity against all isolates of *B. pseudomallei* independent of their LPS phenotype (161). LL-37 also has an essential role in promoting angiogenesis and as a wound healing agent (162). At higher concentrations, LL-37 induces the production of cytokines and chemokines, and at physiological concentrations LL-37 alters IL-8 production by keratinocytes and bronchial epithelial cells in response to inflammatory conditions (163). Thus, LL-37 plays an important role in epithelial innate immunity (152). In addition to its antimicrobial activity, reduction or excess production of LL-37 has been associated with the skin conditions, atopic dermatitis and rosacea, respectively (164,165).

Indolicidin and **tritrpticin** are tryptophan-rich antimicrobial peptides that also belong to the cathelicidin family (166,167). Indolicidin and tritrpticin are expressed in neutrophils (168) of cow and pigs, respectively. Unlike the α-helical peptides previously discussed, these tryptophan-rich peptides have an extended wedge shape conformation in hydrophobic environments such as the plasma membranes (166,167,169). Indolicidin is a 13-amino acid peptide (ILPWKWPWWPWRR) that contains 5 tryptophans (39%) and 3 prolines (25%) and its C-terminal amino acid amidated. In addition to its high activity against *S. aureus* and *E. coli* (167), indolicidin has potent antifungal activity against *C. neoformans* (MIC, 2–4 μg/ml) and good to moderate activity against *Candida* spp. (MIC,8–32 μg/ml) with the exception of *C. guillermondii* (MIC>32 μg/ml) (160). In addition, Ahmad and colleagues reported that incorporation of indolicidin within liposomes minimized its toxicity, enabling higher dosages to be administered to cure mice with systemic infections of *A. fumigatus*; in contrast, maximal tolerated dosages of free indolicidin did not cure mice of such infections (170). Although an important mechanism for killing microbes may be its action as a membrane ionophore, two studies have suggested that the DNA of the microbe is also an important target of indolicidin (115,116).

Tritrpticin is also a 13-amino acid peptide (VRRFPWWWPFLRR) containing 3 tryptophans (23%), 4 arginines (30%), and 2 prolines (15%). Although no *in vivo* studies have been done to test the efficacy of tritrpticin, Lawyer et al determined that this peptide had weak activity toward *C. albicans* (MIC, 1000 μg/ml) and *A. fumigatus* (250 μg/ml) (166) Unlike the C-terminal amino acid of indolicidin which is amidated, the C-terminal amino acid of tritrpticin is not. When the terminal amino acid of tritrpticin is amidated, antibacterial activity increases

2- to 8-fold while hemolytic activity decreases significantly (171); it is not known whether this increased antimicrobial activity extends to killing fungi. Interestingly, Yang et al found that prolines in tritrpticin were essential for microbial selectivity in that replacing them with alanine markedly increased hemolysis (169).

PAF26, a penetratin-like hexapeptide with the sequence Ac-RKKWFW-NH_2, was identified as a lead candidate against phytopathogenic fungi after screening a combinatorial library of hexapeptides. The MIC of PAF26 against *Penicillum digitatum*, a fungus that causes postharvest decay in fruits, is 4μM. Munoz and colleagues recently found that addition of tryptophan to the N-terminal amino acid increased its activity against yeast (MIC, *S. cerevisiae*, 16 μM) and gram-negative bacteria (MIC, E. coli, 4 μM) (172). PAF26 and its analogs showed little to no hemolysis at 100 μM. In addition, PAF26 has been fused with magainin to augment their antimicrobial activity (173).

Kaxins are synthetic cationic antimicrobial peptides that have a non-amphipathic hydrophobic core segment (174,175). By inserting lysines at the N-terminal end and creating D-enatiomers peptides, Burrows and colleagues developed kaxins with potent candidacidal activity with little lysis. One kaxin, dF21-10K (kkkkkkkkkkaafaawaafaa-NH2), showed MIC between 16 and 64 μg/ml against all fluconazole-sensitive and resistant *Candida* spp. and strains (*C. albicans, C. dubliniensis, C. glabrata, C. guillermondii, C. krusei, C. lusitaniae, C. parapsilosis* and *C. tropicalis)* (175). Notably, dF21-10K showed marked activity with complete killing against biofilms formed by *C. albicans* or *C. tropicalis.* Although elimination of biofilms by antifungal molecules usually requires concentrations 30–2000 times more than their MIC (100), dF21-10K eradicated biofilms at only 10 times their MIC (175).

Cyclic peptides

The vast majority of broad spectrum cyclic antimicrobial peptides contain between 1 and 4 disulfide bonds and adopt β-sheet enriched structure including beta-hairpin, beta-sheet, or alpha-helix/beta-sheet mixed structures. Most of these AMP contain open-ended cyclic structures formed by internal disulfide bonds but θ-defensins, in addition to internal bonds, form closed-ended cyclic structures. With peptides such as defensins that contain multiple disulfide bonds, formation of the correct bond remains a challenge for developing peptide technologies.

Thanatin is an inducible and nonhemolytic 21-amino acid peptide isolated from the insect, *Podisus maculiventris.* Compared to other arthropod AMPs, thanatin has broad spectrum antimicrobial activity. Thanatin has a single disulfide bond forming a C-terminal loop that possesses a strong positive charge. Although it has little homology with other arthropod AMP, thanatin does have homology in its amino acid sequence, structure, and biological function with **brevenins** found in frog secretions. Thanatins are fungicidal against several phytomycotic diseases (e.g., MIC, *N. crassa*, Botrytis cinerea, Nectria haematococca, Trichoderma viride, Alternaria brassicola, and Fusarium culmorum < 5 μM), and two pathogenic fungi in humans (MIC, A. fumigatus, 10–20 μM; T. mentagrophytes, 20–40 μM) (176). These peptides have no activity against yeasts such as S. cerevisiae or C. albicans. Interestingly, close homologs of thanatin isolated from skin secretions of frogs do have activity against S. cerevisiae and C. albicans (MIC, Brevinin-1E, 4.7 μM) (177)

Protegrins are approximately 2-kD cysteine-rich β-sheet peptides found in neutrophils of pigs. Similar to LL-37 and indolicidin, protegrins belong to the cathelicidin family of peptides, but unlike these two linear peptides, protegrins are cyclic antimicrobial peptides. Protegrins contain 16–18 amino acids and have 2 disulfide bridges which are essential for their antimicrobial activities, especially at physiological salt concentrations (178,179). In contrast to defensins, protegrins display full antimicrobial activity in the presence of physiologic saline (180). The

ability to maintain activity in the presence of salt is critical for AMP to exhibit their antimicrobial effects systemically or perhaps via aerosolized therapy. Although protegrins have limited homology with defensins, they share significant homology with **tachyplesins** found in hemocytes of horseshoe crabs (181,182). Protegrins display broad spectrum activity against bacteria, fungi, protozoa, and viruses (20,179,183–186) and their primary mechanism of microbial action is due to lysis of the microbial membrane (187). Cho and colleagues examined the anticandidal activity of protegrins 1–5 (188), and found that the protegrins 1–3 and 5 had greater anticandidal activity (MIC range, 2.50–2.85 μM) compared to protegrin 4 (MIC, 4.78 μM). In addition, a D-enantiomer of protegrin-1 had slightly greater activity compared to protegrin-1. Other analogs of protegrin have been made with either greater antimicrobial activity and/or with decreased hemolysis, but these analogues have not been tested against fungi.

Mammalian defensins are a family of cationic peptides containing 6 highly conserved cysteine residues with 3 disulfide bridges (189,190) that are divided into three subfamilies: α-, β-defensins are found in many mammalian species and θ-defensins are found in Rhesus macaques. The α- and β-defensins differ in amino acid sequence and in the location of disulfide bonds, but their 3-D structures are virtually identical; they both contain 3 antiparallel β-sheets and one α-helix (191). Whereas mammalian defensins usually have antimycotic properties against *C. albicans* (192,193), there are distinct variations in their antifungal activities.

α-defensins have between 29 and 36 amino acids in the mature peptide with 3 disulfide bridges between cysteines 1–6, 2–4 and 3–5. Four α-defensins (**human neutrophil peptides 1–4**; HNP1-4) are constitutively expressed in human neutrophils (30,194–196), and two other α-defensins, HD 5 and 6, are expressed in specialized epithelial cells (e.g., Paneth cells) of the intestines and female urogenital tract (197–199). In addition to membrane “pores” as a mechanism for microbial inhibition, HNP-1 and HNP-2 are able to inhibit protein, RNA, and DNA synthesis (200,201). Lehrer et al. compared HNP1-3 defensins for their anti-candidal activity and found that HNP-1 was the most effective. Although HNP-3 at a concentration of 50 μg/ml showed little to no inhibition of *C. albicans*, HNP-1 greatly reduced the number of colony-forming units and was about 10-fold more effective than was HNP-2 (193). Furthermore, α-defensins from rabbits, **NP-1**, **NP-2**, and **NP-3**, are highly effective against *C. albicans* (193). Indeed, NP-1 had 10-times greater candidacidal activity compared to HNP-1. In addition to *Candida* spp, the rabbit defensins killed *A. fumigatus* (minimal fungicidal concentration, NP-1, 25 μg/ml, NP-2, 50 μg/ml, and NP-3, 100 μg/ml) as determined by a MTT assay (202), and both rabbit NP (MIC, NP-1, 3.75 to 15 μg/ml) and human HNP inhibited the growth of *C. neoformans* (203,204). Because NP-1 and 2 defensins bind tightly to chitin and its fragments, this interaction between these peptides and the cell wall of fungi may be important in their cidal activity (202).

β-Defensins are generally larger than their α-counterparts and contain 38–47 amino acids with disulfide bonds between cysteines 1–5, 2–4 and 3–6. The human β-defensin (HBD) family consists of at least 6 members. **HBD-1**, **HBD-2**, and **HBD-3** are mainly produced by epithelial cells and have an important role in immunity of mucosal and body surfaces (205–210). In contrast to HBD 1–3 which are present in many tissues, HBD-4 is found primarily in the gastric antrum and testes (211) and HBD-5 and 6 are specifically expressed in the epididymis (212). β-Defensins such as HBD-1 may be expressed constitutively (209,213) or those, including HBD-2 or -3 may be induced by various proinflammatory mediators (e.g., TNF- α, IL-1 β) (214–221). Whereas the antibacterial activity of HBD1-4 has been studied by several groups, antifungal studies of β-defensins has been limited to the inducible HBD-2 and HBD-3 peptides (222). Although there was a strong correlation between HBD-2 and 3 in their inhibition of *Candida* spp., HBD-3 was generally more effective. The MIC of HBD-2 and -3 for three isolates of several *Candida* spp. was as follows: *C. albicans* (MIC range: HBD2-- 4.6–

59.2μg/ml; HBD3-- 2.8–7.1μg/ml), *C. tropicalis* (MIC, HBD2--3.9–13.1 μg/ml; HBD3--3.3–14.4 μg/ml), *C. parapsilosis* (MIC, HBD2-- 9.3–17.8μg/ml; MIC, HBD3--1.4–12.4 μg/ml), *C. krusei* (MIC, HBD2--12.2 >250μg/ml; HBD3--2.0–13.7 μg/ml), *C. glabrata (*MIC, HBD2--22.7 >250 μg/ml; HBD3-- 33.8 >250μg/ml) (222). Notably, HBD-3 inhibited the growth of the 3 isolates of *C. krusei* while 1 of the 3 isolates was resistant to HBD2. **Bovine tracheal antimicrobial peptide** (TAP), an analog of HBD-2 that is secreted by respiratory epithelial cells, inhibits the *C. albicans* (MIC, 400 μg/ml (223) vs. MIC, 6–12μg/ml (224)) and the hyphal form of *A. fumigatus* (400 μg/ml) (223). The discrepancy of MIC may be due to differences in the strains of *C. albicans* or in different growth media. In addition to their direct antimicrobial properties, β-defensins can modulate the function of immune competent cells (23).

θ-defensins: Three θ-defensins (rTD-1, rTD-2 and rTD-3) were isolated from extracts of granulocytes from Rhesus monkeys (20). These defensins are circular octadecapeptides that contain 2 anti-parallel β sheets and 3 disulfide bonds (20) that are formed by post-translational splicing of 2 nonapeptides which are derived from α-related defensin precursors (20). θ-Defensins possess a wide spectrum of salt-independent antimicrobial activity against bacteria, viruses (both enveloped and nonenveloped), and fungi. Although they share similar sequence and structural homology with protegrins, they induce significantly less hemolysis. Interestingly, θ-defensins differ from other AMP in their interaction with membranes which perhaps explain their reduced proclivity to induce hemolysis. The rTD1-3 defensins have significant activity against *C. albicans* (MIC, rTD1,2--1μg/ml; rTD3--3μg/ml) (20,225) and are also active against *C. neoformans* (20). Their activity against other fungi has not been reported.

Poultry **gallinacins**, produced by leukocytes, are rich in arginines and lysines, have 3 disulfide bonds, and are functional and structural equivalents to mammalian β-defensins (226). While gallinacin-1 and 1 αinhibited *C. albicans,* gallinacin-2 did not (226). Strong bactericidal and fungicidal activities (MIC, 8 μg/ml, *C. albicans, S. cereviseae*) were observed for gallinacin-6, which is expressed in the digestive tract of chickens (227). Because of the potential resistance developing in microbes exposed to low levels of antibiotics, there has been increasing interest in stimulating endogenous AMP such as gallinacin 6 to suppress microbial growth in chickens.

Macrocyclic peptides, isolated from coffee plants, contain 4 end-to-end large cyclic peptides with 6 disulfide bonds (228). Four macrocyclic cystine-knot peptides of 29–31 residues, sharing 45% homology with one another, belong to this family (kalata, circulin A and B (CirA and CirB), and cyclopsychotride (cpt)). In contrast to kalata and CirA, which are relatively ineffective against gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa,* CirB and cyclopsychotride kills both gram-positive and gram-negative bacteria, in particular *E. coli*. Although the cyclic peptides were inactive against *C. albicans*, all peptides in a low salt assay were moderately active against *C. kefyr* (MIC range-14.0 to 29.0 μM) and two of the four peptides were active against *C. tropicalis* (MIC, CirA, 21.4μM; Cpt, 56.5μM (228). The antifungal and antibacterial activities of these peptides were significantly diminished when 100 mM NaCL is added to the assay medium. Unless improved analogs are discovered, macrocyclic peptides will likely have a limited role as antifungal agents.

Syringomycins and related peptides are a group of cyclic lipopeptides, called lipodepsinonapeptides, produced by *Pseudomonas syringae*. This group of peptides induces ion channels that affect membrane function, including membrane potential, protein phosphorylation, and H+-ATPase activity (229,230). The family members (syringomycin E, syringotoxin B, and syringostatin A) were effective *in vitro* against a number of isolates of *Candida* spp. *(*MIC, 2.5–25 μg/ml), *C. neoformans* (MIC, 0.8–10 μg/ml) and *A. fumigatus* (MIC, 5–40 μg/ml) (231). Generally, the 3 cyclic peptides were more effective against yeasts than against filamentous fungi. Moreover, syringotoxin B was the least effective antifungal

cyclic peptide. In a subsequent study, syringomycin E was found also to be effective in treating vaginal candidiasis in mice (232).

ANTIMICROBIAL PEPTIDES THAT ARE PROTEOLYTIC FRAGMENTS OF PROTEINS

The majority of peptides derived from proteins such as lactoferrin, BPI, and pepsinogen A have broad-spectrum antimicrobial activity. For example, hydrolysates of lactoferrin have marked activity against gram-negative and gram-positive bacteria (233). Depending on the location of the protein in which the peptides were derived, the peptides may have an α-helical or β-sheet structure. Moreover, these proteolytic peptide fragments frequently have greater antifungal activity than the parent proteins. Although we discuss mammalian peptides from lactoferrin and BPI in this review, amphibian antimicrobial peptides derived from the amino-terminal domains of Pepsinogen A and C have also been identified (234).

Lactoferrin is an iron-binding antimicrobial glycoprotein (78 kDa) that is present in neutrophil granules, in breast milk, and on many mucosal surfaces. (235,236). Lactoferrin is multifunctional and can influence a wide range of biological processes including immune responses. There are two mechanisms by which lactoferrin is thought to inhibit fungal and bacterial growth: 1) by depriving iron which is essential to growth of fungi and 2) generation of antifungal peptides from proteolytic enzymatic digestion of lactoferrin. Kirkpatrick et al. initially proposed that the iron-binding properties of lactoferrin were responsible for its inhibition of *C. albicans* (237). Later, Zarember and colleagues showed that iron sequestration had an important role in the inhibition of *A. fumigatus* by lactoferrin (238). A recombinant lactoferrin (talactoferrin, Agenix) is now in clinical trials for treatment of cancer, diabetic skin ulcers, and sepsis. Enzymatic degradation of lactoferrin results in several antimicrobial/antifungal peptides (lactoferricin H, B; peptide 2; kaliocin-1) (239–242). Except for kaliocin, the antimicrobial peptides are derived from the N-terminal region of lactoferrin. Many of these proteolytic peptides posses stronger antimicrobial activity than did lactoferrin itself (233). The N-terminal lactoferrin-derived peptides that lack the iron-binding domain of the parent protein exert their antifungal activity by affecting permeability of the membrane (243). After the N-terminal amino acid lactoferrin H peptide was first identified, several smaller peptides have been identified with antifungal activity. Viejo-Diaz et al. identified Lfpep (residues 18 to 40 of human lactoferrin) that were candidicidal against fluconazole and amphotericin-resistant *Candida* spp. (MIC range, 9.3 to 18.7 μM) (241). Another well known antifungal peptide derived from lactoferrin is peptide 2 (242). Ueta and colleagues compared six N-terminal peptides derived from lactoferrin and found that peptide 2 (FKCRRWQWRM) had the most antifungal activity; in two strains of *Candida*, the MIC of Peptide 2 was approximately 17 μM, which was close to the MIC of miconazole. Interestingly, replacing the single cysteine of peptide 2 with alanine markedly mitigated its anticandida activity. Although Peptide 2 cannot form a cyclic structure, perhaps the single cysteine enables dimers to form, which may be more potent antifungal agents. Moreover, peptide 2 augments the candidacidal activity of polymorphonuclear leukocytes by inducing superoxides (242).

BPI protein domain III analogs

The bactericidal and permeability-increasing (BPI) protein is a 55-kDa cationic protein that is stored in azurophilic granules of neutrophils. BPI inhibits gram-negative bacteria by binding LPS with high affinity and it also shows antifungal activity against *H. capsulatum* (MIC50: 1 μg/ml; MIC, 80–90 μg/ml) (244). Moreover, BPI together with defensins and cathepsin G are additive in their inhibitory activity against *H. capsulatum* (244). Compared to the parent protein, proteolytic fragments of BPI have significantly more antifungal activities directed towards *Candida* spp., *C. neoformans*, and *A. fumigatus* (245). On the basis of the BPI protein

domain III structure, three synthetic peptides (XMP.284, XMP.366, XMP.391) have been reported to be effective against several *Candida* species and showed synergistic activity with fluconazole (246). Additionally XMP.391 was found to increase the antifungal activity of amphotericin B against systemic aspergillosis in a murine model (247).

SYNTHETIC STRATEGIES FOR PRODUCING PEPTIDES FOR PRECLINICAL AND CLINICAL APPLICATIONS

Naturally occurring broad-spectrum antimicrobial peptides and their analogs have been examined for their utility as therapeutic antibiotics by many groups, and the ability to produce AMP economically is a key factor that will determine which AMP will receive widespread use for treatment of fungal infections. To date, the only approved antifungal peptides are in the echinocandin family (micafungin, caspofungin, and anidulafungin); precursors of these FDA-approved peptides are isolated from various molds and further modified chemically. Nonetheless, depending on the original source of the peptide, isolation of natural compounds can often be prohibitively expensive and peptide isolates may be sensitive to protease digestion. In many cases, peptide technologies including solid-phase peptide synthesis and recombinant-engineering methods provide mechanistic insight about the peptide and offer the potential of creating more effective antifungal therapies in a cost-saving manner.

Several groups have synthesized peptides by a solid-phase peptide method for their antimicrobial and antifungal studies. The synthesized peptides have included linear (170, 223,225,248,249) and cyclic peptides with one to multiple disulfide bonds (76,228,250,251) and closed-ended cyclic theta defensins peptides (225). Synthesized peptides have ranged in size from 6 (the hexapeptide) to 31 residues. Solid phase peptide syntheses are ideally suited for structure-function studies because the amino acid sequences can rapidly be altered to compare their antimicrobial activities with the parent peptide. For example, Chen and colleagues synthesized 20 analogs of protegrins to gain understanding of the relationship between the primary and secondary structure and the antimicrobial activity (252). From these structural activity relationship (SAR) studies, the IB-367 analog was identified for further clinical investigations. Several such SAR studies have been done to identify analogs with increased efficacy and reduced toxicity (176). In addition to the SAR function studies, several hybrid peptides (e.g., cecropin A-magainin, HP ribosomal peptide-magainin; cecropin A-mellitin) have been made by peptide synthesis and have increased biological activity toward microbes compared to either peptide alone (134,135,253). For clinical studies, it is likely that peptide synthesis will prove cost-effective in AMP production for smaller peptides, approximately 25 amino acids or less. Of note, the length of peptide that can be synthesized without significant truncation depends on its amino acid sequence and content. Thus far, analogs of protegrins, histatins, and indolicidin have been made for clinical trials by peptide synthesis (254). Therapeutic peptides with increased length and complexity continue to be made. Improvements in synthesis and development of higher-quality resins for solid-phase synthesis, price reductions for commonly used reagents, and increased competition are enabling efficient and cost-effective production of these peptides. Peptide synthesis is an evolving methodology in which further advances to their production are anticipated.

Recombinant engineering is an alternative technology that has been used to synthesize various antimicrobial peptides (255–260). There is significant overlap in the antimicrobial studies that have been done with recombinant-engineering and solid-phase synthesis technologies. For example, SAR studies and hybrid antimicrobial peptides have also used recombinant engineering methods (133,173,261). Recombinant engineering is more appropriate and cost-effective for larger AMPs such as defensins, which have between 30 to 50 amino acids and 3 or more disulfide bonds. Several organisms have been used to synthesize recombinant antimicrobial peptides, including *E. coli*, baculovirus insect cells, fungi (*Pichia Pastoris, S.*

cerevisiae, *Aspergillus* spp.) and chloroplast expression systems (262); thus far, expression in *E. coli* and fungi have yielded promising results. Fusion proteins produced in *E.coli* are the most widely used in recombinant engineering because of their low cost and the large amount of information on the strains and available plasmids (255). Many antimicrobial peptides are toxic to bacteria so that these peptides are often fused with proteins such as thioredoxin and glutathione-S-transferase (255,263); in addition to preventing cell lysis, thioredoxin and glutathione-S-transferase aid in disulfide bond formation of the antimicrobial peptide (264). Although most expression plasmids produced low levels of antimicrobial peptides in *E. coli*, the IPTG-inducible pET-32a(+) expression vector produced levels of HBD-2 reaching 1.3 g/l (265). To express antifungal peptides in yeast and filamentous fungi, resistant strains of these organisms that lack the target to these peptides have been developed. In *P. Pastoris*, the plant defensin, Psd1, fused with MFa1 signal peptide and a pro-region produced fusion protein levels of 60 mg/L. Expression systems in filamentous fungi have yielded even higher levels of proteins (10 g or more/L). Talactoferrin, which has entered phase III clinical trials for treatment of foot ulcers, sepsis, and cancer, is expressed by *Aspergillus* spp.

Another synthetic strategy to develop cost-effective antibiotics is to utilize antimicrobial peptide mimetic agents. For example, Beckloff and colleagues developed meta-phenylene ethynylene (mPE) compounds that have an amphipathic structure similar to magainins. mPE exhibited antimicrobial activity at nanomolar concentrations against a variety of bacteria and *Candida* spp. found in oral infections (266). Other magainin-mimetic compounds such as MSI-751 have also been developed (267). Electron microcopy showed formation of ion channels in pathogenic organisms with these mimetics that was similar to magainins. Moreover, experimental studies to identify the determinants underlying the configuration of these peptides will likely provide adequate information for computer simulation methods to explore their fungicidal mechanism at the atomic level. Interactions between peptides and lipid bilayers are being investigated by molecular dynamics (MD) methods (268). By understanding structural and dynamic properties of peptides and their target, MD-based methodology increases the likelihood for rational design of antimicrobial peptidomimetics (269,270).

CONCLUDING REMARKS

Both plants and animals are constantly exposed to harmful pathogens present in all environmental niches. Moreover, some organisms lack components related to specialized immunity such as lymphocytes or antibodies. Nonetheless, living organisms have found a way to live and thrive. During the evolutionary process, nature was able not only to create but also strategically localize small antimicrobial peptides with relatively simple modes of action, resulting in survival of life forms on this planet. The pharmaceutical use of these antimicrobial peptides and analogs may also have an important role in treating human infectious diseases because of increased resistance to the widespread use of currently-used antibiotics. Whether resistance to these antimicrobial peptides will occur has been debated, but it is likely that resistance will occur at a reduced rate and will depend on how the antimicrobial peptide is administered. Combination therapy of the antimicrobial peptide with antibiotics or with other peptides will likely reduce the development of resistance markedly. One area that merits further attention and research is the use of cationic peptides to prevent the formation of polymicrobial biofilms. Although there are promising peptide candidates that have demonstrated marked reduction of biofilms caused by bacteria or *Candida* species (98,175,266,271–273), we are not aware of studies demonstrating the efficacy of these peptides against biofilms caused by multiple organisms (274). Regardless of the effectiveness of naturally occurring peptides as antifungal agents, new peptide strategies offer the potential of developing more potent, specific and non-toxic therapies. Several examples given in this review have shown that modified peptides are more effective than their parent peptides and most AMP in clinical trials are analogs of the parent peptide.

Current antimycotic therapies face many difficulties including fast growing drug-resistance, limited choice of antifungal drugs, and increased fungal infections in immunosuppressed patients with AIDS, organ transplant or cancer. Candidemia in these patients leads to an overall mortality of 40% in the United States (275). Although AMP and their analogs bring the hope of treating these serious antifungal infections more effectively, interactions of new therapies with biological pathways to address safety concerns will need to be addressed. To date, the only antifungal peptides to receive FDA approval are the echinocandin family of β-glucan inhibitors; these inhibitors have been approved for treatment of candidemia, empiric treatment of neutropenic febrile patients, and invasive aspergillosis. In addition, there are a number of other antifungal/antimicrobial peptides in various stages of preclinical and clinical trials, primarily involving topical and aerosolized approaches to infections (see reviews by Gordon (254) and Jenssen and Hancock (40)). With the many promising antifungal peptides that show selective toxicity toward pathogenic fungi, we anticipate that peptide analogs will continue to be developed with increased therapeutic efficacy and have an important role in treating patients with fungal infections.

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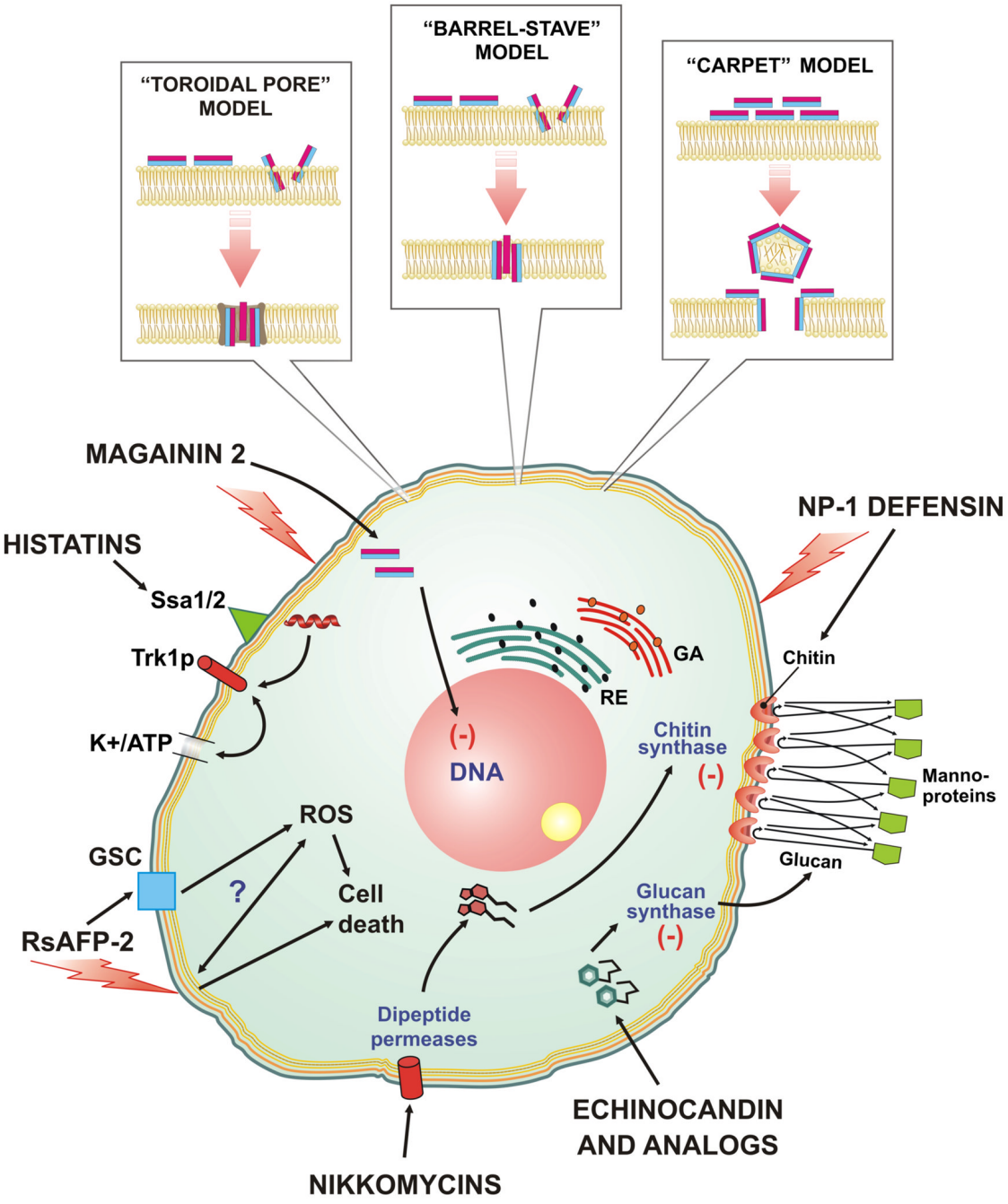

Figure 1. Modes of action proposed for anti-fungi peptides

The majority of anti-microbial peptides inhibit filamentous fungi and yeasts by membrane lysis: three mechanisms (the toroidal pore, barrel-stave, and carpet) have been proposed to induce lysis of the membranes by AMP. In addition, there are other AMP that are more selective in their antifungal activity. Several anti-fungi peptides disrupt function and/or structure of the cell wall. Defensin NP-1 binds tightly to chitin in the cell wall and this may be important in mediating selective membrane lysis. In addition, chitin biosynthesis is blocked by nikkomycins which enter fungal cells via dipeptide permeases. The lipopeptide echinocandin and its synthetic analogs inhibit synthesis of 1–3 β glucans, a key component of the cell wall. For histatins, one mechanistic concept is based on specific binding with extracellular receptor like

Ssa1/2, internalization, and binding to K+ channel transporter Trk1p and as a consequence, efflux of ATP and potassium ions from the cell. MAG-2 magainin kills fungi via cell membrane disruption and DNA damage. The plant defensin, RSAFP-2, causes membrane permeabilization via interaction with glucosylceramide (GSC) and formation of reactive oxygen species (ROS). M – mitochondria; N – nucleus; E – endosomes; L – lysosomes; RE – endoplasmic reticulum; GA – Golgi apparatus.

Table 1

Antimicrobial peptides that specifically target fungi

Name of the group	Representative peptides	Origin	Structure	Mechanism	MW[1]	Susceptible strain	References
β-glucan synthase inhibitors	Echinocandins Pneumocandins Aculeacins	Fungi	Cyclic lipopeptides	Inhibition of glucan synthesis	1000	*Candida spp. P.carinii Aspergillus spp*	Benz F et al, 1974 (47); Fromtling R et al, 1989(50); Iwata K et al, 1982 (58)
	Mulundocandins		Threonine is substituted by serine; and lineoyl with 12-methylmyristoyl				Roy K et al, 1987 (60); Hawser S et al, 1999(61)
Cell wall chitin inhibitors	Nikkomycins Polyoxins	Bacteria	Nucleoside peptide antibiotic	Inhibition of chitin synthesis	Nikkomycin Z 495	*Candida spp. Coccidiodes immitis Balstomyces dermatides Histoplasma capsulatum*	McCarthy P et al, (66); Hector R et al, 1990(67)
	Aureobasidins	Fungi	cyclic lipophilic depsipeptide with 8 amino acids and an-hydroxyacid	Inhibition of actin and chitin assembly; synthesis of sphingolipids	1100	*Candida spp C. neoformans.*	Ikai K et al, 1991 (69); Endo M et al, 1997(70); Nagiec M et al, 1997(71)
Membrane-targeting inhibitors	Rs-ARF2	Plants	α-helical 3-stranded β-sheets, 4 disulphide bridges	Target membrane glycosylcerebroside, induces reactive oxygen species, membrane lysis (?)	5730	*Candida albicans, C. krusei, A. flavus, Fusarim solani.*	Schaaper W et al, 2001(76); Thevissen K et al, 2007(73)
	Drosomycin	Insects	α-helical 3-stranded β-sheets, 4 disulphide bridges	Target voltage- gated sodium channel (?), Membrane lysis (?)	5250	*F. oxysporum N. crassa S.cerevisiae*	Fehlbaum P et al, 1994(74); Bulet P et al, 2005(276); Yuan Y et al, 2007 (277); Cohen L et al, 2009(77)
	Bacillomycin F Iturin A	Bacteria	Cyclic with lipid-soluble β-amino acid linked to the D/L aa	Lysis by pore formation and leakage of key ions	1000	*Aspergillus niger C.albicans F.oxysporum A. flavus F. moniliforme*	Besson F et al, 1984(81) Mhammedi A et al, 1982(82)
Histatins	Histatin 1–12	Primates	α-helical in hydrophobic environment; increase histidine content	Mechanism uncertain (Target mitochondria vs. Trk1 potassium transporter vs lysis of	Histatin1: 4880; Histatin 3: 4060; Histatin: 5: 3040	*Candida spp. Trichosporon pollulans, Cryptococcus*	Pollock J et al, 1984(88); Helmerhorst E et al, 1999(90); Tsai H et al, 1997(91); Baev D et al, 2004 (108); Mochon A et al, 2008(105);

Name of the group	Representative peptides	Origin	Structure	Mechanism	MW[1]	Susceptible strain	References
				energized membrane)		*neoformans*, *A. fumigatus*,	Troxler R et al, 1990(278)

[1]-Approximate molecular weight

Table 2

Broad-Spectrum Antimicrobial Peptides

Name of the group	Representative peptides	Origin	Structure	Mechanism	MW[1]	Susceptible strain	References
Cecropins	60 members; Cecropin A	Insects	α-helical Tryptophan in the first or second position; C-amidated end	Membrane lysis	2800–4000	*Aspergillus spp. Fusarium moniliforme Fusarium oxysporum*	Giacometti A et al, 2001; (121) De Lucca A et al, 1988; (124)
Magainins	Magainin 2	Frogs	α-helical Rich in glycine and serine amino acids	Membrane lysis; DNA damage	2500–3000	*C. albicans C. neoformans Saccharomyces cerevisiae*	Zasloff M 1987(111); Morton C et al, 2007 (114)
Bombinin-like and bombinin H	BLP-1,3 Bombinins H1-7		α-helical Glycine-rich; C-terminal amino acid amidated	Membrane lysis	2300–4000	*C. albicans C. guillermondii C. tropicalis*	Simmaco M et al, 2009(137)
Dermaseptins	Dermaseptin S1-5		α-helical Lysine-rich	Membrane lysis; Apoptosis induction	2500–3500	*A. fugimatus*	Mor A et al, 1991(112); Morton C et al, 2007 (114)
Cathelicidins (without cysteines)	LL-37; mCRAMP; BMAP-27–28	Humans, cattle, pigs, goats, mice	α-helical	Membrane lysis	3000–4000	*C. albicans C. neoformans*	Boman H et al, 2003 (279); Benincasa M et al, 2006(160)
	Indolicin; Tritrpticin		Extended wedge Rich in tryptophans	Membrane lysis	1900–2000	*C. neoformans Candida spp. A. fumigatus*	
PA26	PA26	Combinatori al Library	Penatratin-like hexapeptide	Unknown	950	*Penicillum digitatum, S. cerevisiae*	Munoz A et al, 2006 (280)
Kaxins	dF21-10K	Synthetic design	Non-amphipathic hydrophobic core N-terminal lysine-rich	Membrane- lysis	1830–2350	*Candida spp.*	Burrows H et al, 2006 (175)
Thanatin	Thanatin		β-sheet, single disulfide bond forming C- terminal	Membrane lysis	2400	*A. fumigatus N. crassa T. mentagrophytes*	Fehlbaum P et al, 1996(176); Bulet P et al, 1999 (281)
Cathelicidins (with cysteines)	Protegrins1-5	Pigs	β-sheet, 2 disulfide bridges	Membrane lysis	1900–2200	*C. albicans*	Kokryakov V et al, 1993 (282)
α-Defensins	HNP1-4; HD5-6; NP-1	Mammals	Cyclic: 3, Disulfide bridges C1-6, C2-4, C3-5	Membrane lysis	3000–4000	*Candida spp. A. fumigatus*	Ganz T et al, 2002 (30);

Name of the group	Representative peptides	Origin	Structure	Mechanism	MW[1]	Susceptible strain	References
						C. neoformans	Lehrer RI 2004(283); Levitz S et al, 1986 (202)
β-Defensins	HBD1-4 Bovine tracheal antimicrobial peptide	Mammals	Cyclic: Disulfide bridges C1-5, C2-4, C3-6	Membrane lysis	3500–5000	*Candida spp* *A. fumigatus*	Diamond G et al, 1991 (224); Joly S et al, 2004(222)
θ-Defensins	rTD1-3	Rhesus monkeys	Circular octadecapeptides, 2 anti-parallel β-sheets and 3 disulfide bonds	Membrane lysis	2100	*C. albicans* *C. neoformans*	Tang, YQ et al, 1999 (20); Tran, D et al, 2008(225)
Gallinacins	Gallinacin-1 and 1 α, gallinacin-2 and -6	Chicken	β-sheet, rich in lysine and arginine, 3 disulphide bonds	Membrane lysis combined with effect on DNA replication, RNA and protein synthesis	2000–5000	*C. albicans* *S. cereviseae*	Van Dijk A et al, 2007 (227)
Macrocycles	Kalata Circulin A,B Cyclopsychotride	Plants	Cyclic knot	Membrane lysis	2800–3400	*C. kefyr* *C. tropicalis*	Tam J et al 1999(228),
Syringomycins Pseudomycins	Syringomycin E Syringotoxin B Syringostatin A	Bacteria	Lipodepsinonapeptides	Membrane lysis	2300–3500	*Candida spp* *Aspergillus*	Feigin A et al, 1996 (229); Sorensen K et al, 1996 (231)
Lactoferrin[2]- dervived peptides	Peptide 2 Lactoferricin	Mammals	Glycosylated protein, two symmetrical lobes (N and C lobes), mixture of α-helix and β-sheet	Membrane lysis	2600–4660	*C. albicans Crypt. albidus,, Dekkera bruxellensis, Pichia membranifaciens, Saccharomyces cerevisiae, Zygosaccharomyces bisporus*	Gonzalez-Chavez S et al, 2009 (284) Ueta E et al; 2001;(242) Viejo-Diaz, M. et al, 2005 #4524} (241)
BPI protein[2] domain III analogs	XMP.284 XMP. 366 XMP.391		Lipid binding protein; the amino-terminal rich in lysine residues	Membrane lysis	1550	*Candida spp* *C. neoformans* *A. fumigatus* *Histoplasma capsulatum*	Elsbach, P. et al, 2003 (245)

[1]MW, approximate molecular weight;

[2]The molecular weights of lactoferrin and BPI are 55000 and 80000, respectively