Antifungal Peptides with Potential Against Pathogenic Fungi

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Abstract

Systemic fungal infections have increased over time due to the rise in the at-risk population, which includes immunocompromised patients, those submitted to organ transplantation or undergoing chemotherapy. Clinically available antifungals are limited since some of them have important side effects, being toxic to the host cells, and some can quell filamentous fungi, but their activity against pathogenic yeasts is not killing but controlling their multiplication. Antimicrobial peptides are multifunctional molecules expressed by several microorganisms or synthetized by different techniques. They can play a central role in infection and inflammation. Some of their other effects include chemotactic and immunomodulating activities and wound repair. Antimicrobial peptides (AMPs) can be isolated from a large variety of microorganisms, such as plants, vertebrates, insects, bacteria, and fungi. They are classified into categories according to their amino acid composition, size, and conformational structures, and naturally occurring peptides can be synthetized. Solid-phase peptide synthesis allows the use of nonproteinogenic amino acids and permits changes in structural and physicochemical properties. In these terms, peptide engineering is a useful tool to adjust features such as net charge, surface hydrophobicity, and polarity, and it may also optimize activity and overcome the limitations inherent to natural peptides. AMPs have potential applications in antifungal therapeutics in human health, and recent uses of synthetic AMPs against fungal infections are discussed in this article.

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A. Basak et al. (eds.), *Recent Trends in Antifungal Agents and Antifungal Therapy*,
DOI 10.1007/978-81-322-2782-3_3

Abbreviations

ABP-dHC	Antimicrobial peptide drury *Hyphantria cunea*
AIDS	Acquired immune deficiency syndrome
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
BMAP	Bovine myeloid antimicrobial peptides
DNA	Desoxyribonucleic acid
EDMC	Electrostatically driven Monte Carlo
FDA	US Food and Drug Administration
GPI	Glycosylphosphatidylinositol
HIV	Human immunodeficiency virus
hLF	Human lactoferrin
HNP	Human neutrophils peptides
HP	Human defensins
MDR	Multidrug-resistant
MPTP	Mitochondrial permeability forming transition pores
PI	Propidium iodate
PMAP	Porcine myeloid antimicrobial peptide
SMAP	Sheep myeloid antimicrobial peptide

3.1 Introduction

Pathogenic fungal infections are the seventh most common cause of infection-related deaths in the United States, and the fourth cause of nosocomial infection is due to the fungal pathogen *Candida albicans* (McNeil et al. 2001; Fisher et al. 2012; Wisplinghoff et al. 2004). Systemic mycoses can be classified according to whether the causative agent is a systemic fungal pathogen (*Coccidioides immitis*, *Histoplasma capsulatum*, and *Paracoccidioides brasiliensis*) or one of the increasing number of opportunistic fungal pathogens, including *C. albicans*, *Cryptococcus neoformans* var. *grubii* (Maurya et al. 2011; Rodriguez-Cerdeira et al. 2014), and several other ones. Those infections have become more frequent among the increasingly large population of individuals with severe immune deficiencies, including those with human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) (Martinez and Temesgen 2006; Marukutira et al. 2014).

The treatments of those systemic fungal infections are primarily based on itraconazole, fluconazole, or amphotericin B (Rodriguez-Cerdeira et al. 2014; Kahn et al. 2014). The triazole group enhances the specificity for fungal cytochrome P450 target, and the extra methyl group in fluconazole enhances the hydrophobic interactions at the active site, but they present limitations of toxicity or bioavailability problems that affect their potential use as systemic agents (Fukuoka et al. 2003; Ostrosky-Zeichner et al. 2010).

Amphotericin B is used for the treatment of many types of invasive fungal infections and binds to ergosterol to form membrane pores, which leads to leakage of intracellular constituents (Gabrielska et al. 2006). Despite its undeniable antifungal activity, amphotericin B has many side effects, such as nephrotoxicity (Fanos and Cataldi 2001; Wong-Beringer et al. 1998). These side effects occur mainly because drug targets in fungi are homologues of some molecular sites in humans. In addition to this limitation, the development of antimicrobial resistance due to the use of broad-spectrum antifungal drugs and their limited number for clinical purposes is a concern for public health. Considering these facts, research into new molecules with future potential therapeutic application is extremely important. Antimicrobial peptides have potential use as antifungal agents, killing the microorganism directly, or immunomodulating the host immune system response (Maurya et al. 2011; Zhai et al. 2010; Lakshminarayanan et al. 2014; Wong et al. 2013; Steinstraesser et al. 2011; Lim et al. 2015).

3.2 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are naturally occurring molecules that play an important role in the first line of defense against microbial

threats. They can be isolated from organisms as diverse as humans, plants, insects, and even other microorganisms like bacteria (Zasloff 2002). They are produced due to an exposure to infecting microorganisms and act in order to kill or to slow the growth of invading microorganisms and to aid allied mechanisms of natural and adaptive immunity (Fox 2013; Brogden 2005).

AMPs have a broad spectrum of activity against bacteria, fungi, enveloped viruses, parasites, and even cancerous cells. They can act directly on microorganism membranes or other nonspecific cell targets, which is an advantage in avoiding the development of microbial resistance by gene mutation, as it might happen when drugs have specific proteins as targets (Peschel and Sahl 2006). Moreover, those peptides can be extremely variable in length, amino acid composition, and structure (Nguyen et al. 2011). According to their predominant secondary structure, AMPs are divided into four categories: (a) α-helical, (b) β-sheet, (c) mixed α-helix/β-sheet, and (d) extended. The net positive charge of cationic peptides (+2 or +9) mediates their selective activity against microorganisms' cells that carry a negative net charge due to arginine and lysine residues. Those peptides also have approximately 50 % of hydrophobic amino acids that facilitate interactions with the fatty acyl chains (Steckbeck et al. 2014; Hancock and Patrzykat 2002; Garibotto et al. 2010; Hancock and Rozek 2002).

Some of the AMP mechanisms of action involve different membrane interactions. Membrane disruption can occur through the formation of toroidal pores, composed of loosely associated peptides with interdigitating phospholipid head groups among them. Those peptides are at a critical threshold concentration (Brogden 2005). Differently, in the "barrel-stave model," the peptides are not associated with the lipid head groups, but their hydrophobic regions align with the lipid core region of the bilayer, and the hydrophilic peptide regions form the interior region of the pore (Yang et al. 2001). Another type of membrane interaction is through peptide accumulation on the bilayer surface, since they are electronically attracted to the anionic phospholipid head groups at numerous sites, thus covering the surface of the membrane in a carpet-like manner. At high peptide concentrations, these surface-oriented peptides may act like detergents, leading to the formation of micelles (Shai 1999; Ladokhin and White 2001).

The mechanism of action for the antifungal activity of peptides is generally more complex and often involves entry of the peptide into the cell. It occurs mainly because of the fungal cell architecture, briefly described since they are targets for antifungal drugs (Yu et al. 2014). The cell wall has been shown to be primarily composed of chitin, glucans, and glycoproteins, all of them being covalently cross-linked together. The glycoproteins presented in the cell wall are extensively modified with both N- and O-linked carbohydrates and, in many instances, contain a glycosylphosphatidylinositol (GPI) anchor as well (Bowman and Free 2006). The β-glucan network consists largely of (1–3)-β-glucans with (1–6)-β-branches. In yeast (1–6)-β-glucans are also present. The cell wall is also composed of glycosylated proteins that can be decorated with mannose, galactose, glucose, and uronic acid residues. Chitin and (1-3)-β-glucan layer are a target for a wide range of antifungal molecules that can affect their synthesis and lead to a growth inhibitory effect (Fontaine et al. 2000; Schoffelmeer et al. 1999; Theis and Stahl 2004). Figure 3.1 is a schematic representation of some possible peptide membrane interactions and the basic fungi cell wall structure. Fungal plasma membranes are composed of three main lipids including phospholipids, sphingolipids, and sterols, mainly ergosterol (differently from mammalian cells, composed of cholesterol). The difference in sterol content has been exploited in the mechanism of antifungal drugs such as amphotericin B and azoles (van der Weerden et al. 2013; Kaminski 2014). The mechanisms through which AMPs have antimicrobial activity involve not only membrane physiology interference or disruptions (as described by the proposed models of barrel-stave, carpet, or toroidal pores). They might also interact with protein targets associated with the membrane or by intracellular

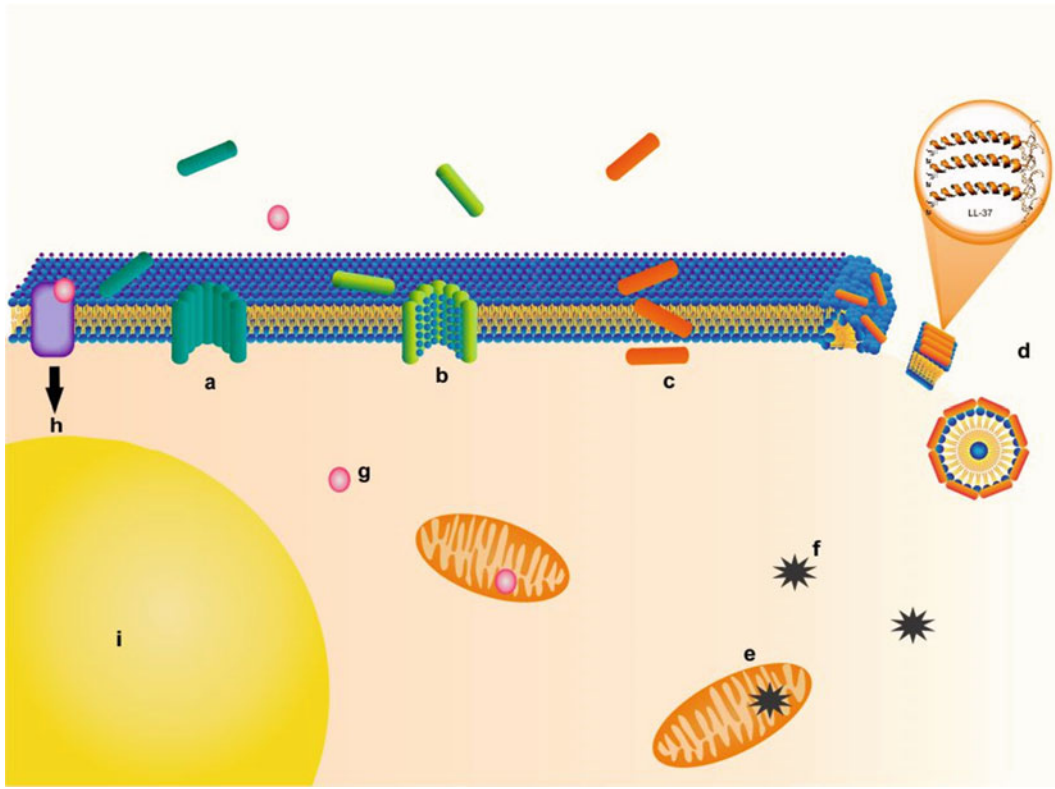

Fig. 3.1 Peptide interaction with the fungal membrane. Schematic representation of the fungal cell wall, composed of outer protein layer with carbohydrate residues (*dark purple*). Peptides with hydrophilic head group and hydrophobic acyl side chain regions (*dark green*, *light green*, and *orange*) interacting with fungal plasma membrane as barrel-stave pore (**a**), toroidal pore (**b**), or by membrane translocation (**c**) and membrane disruption in a carpet-like manner (**d**), as highlighted for LL-37. These interactions depend on the peptide, its concentration, and lipid composition of the membrane. Some peptides such as histatins, β-defensins, lactoferricins, RK-31, KS-30, and hLF (1-11) can reach internal targets such as the mitochondria (**e**). Histatin 5 also leads to the generation of (**f**) reactive oxygen species – ROS (*black spindles*). The non-lytic release of ATP (*pink*) (**g**) by HNP-1, HNP-2, HNP-3a, histatin-5, hLF(1–11), hLF(21–31), and B4010 might activate cell death pathway and (**h**) induce G1 phase arrest of the nucleus (**i**)

targets, including DNA and protein synthesis, protein folding, enzymatic activity, and cell wall synthesis, which may confound the generation of resistance development (Lakshminarayanan et al. 2014; Brogden 2005; Jenssen et al. 2006; Hale and Hancock 2007; Hancock et al. 2012; Yount et al. 2006). As an example, histatins bind to a receptor in the fungal cell membrane, enter the cytoplasm, and induce the non-lytic loss of ATP from actively respiring cells. Their action can also disrupt the cell cycle and lead to the generation of reactive oxygen species (Kavanagh and Dowd 2004; De Smet and Contreras 2005) (Table 3.1).

The isolation and characterization of a natural peptide is a long and laborious process that can hinder the clinical use of AMPs. A new approach is the design of synthetic sequences, which are the result of optimizing sequence and chemical characteristics that are common to many types of AMPs (pharmacophoric patron). Ideally, an antifungal peptide agent should be as short as possible, and therefore the de novo peptide design approaches help to minimize costs production and can help to overcome the low in vivo activity, the labile nature of peptides, and potential toxicity (Steckbeck et al. 2014; Garibotto et al. 2010).

Table 3.1 Antifungal peptides

Antifungal peptides				
Origin	Name	Species	Effective	References
Insect	Alo-3	*Acrocinus longimanus*	*Candida albicans*, *C. albicans* ATCC 90030, *C. glabrata*, C. glabrata ATCC 36082	van der Weerden et al. (2013) and Barbault et al. (2003)
	Termicin	*Pseudocanthotermes spiniger*	*C. albicans*, *Cryptococcus neoformans*	Da Silva et al. (2003) and Lamberty et al. (2001)
	Holotricin-3	*Holotrichia diomphalia*	*C. albicans*	Lee et al. (1995)
	Tenecin-3	*Tenebrio molitor*	*C. albicans*	Kim et al. (1998)
	Cecropin A	*Hyalophora cecropia*, *Drosophila*	*Aspergillus fumigatus*	Steiner et al. (1988) and De Lucca et al. (1997 2000)
	ABP-dHC-cecropin	*Hyphantria cunea*	*C. albicans*, *Neurospora crassa*, *Rhyzopus*, *Fusarium*, *Alternaria*, *Mucor*	Zhang et al. (2015)
	Rondonin	*Acanthoscurria rondoniae*	*C. albicans*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*	Riciluca et al. (2012)
Amphibian	Brevenin-1BYa	*Rana boylii*	*C. albicans*, *Staphylococcus aureus*	Conlon et al. (2003), Yeaman and Yount (2003), and Pal et al. (2006)
	Brevenin-1Pa, brevenin-1Pb, brevenin-1Pc	*Rana pipiens*	*C. albicans*, *S. aureus*, *Escherichia coli*	Goraya et al. (2000) and Marenah et al. (2004)
	Temporin A	*Rana temporaria*	*C. albicans*	Mangoni et al. (2000)
Mammalian	Indolicidin	Cytoplasmic granules of neutrophils	*C. albicans*, *C. neoformans*, *S. aureus*, *E. coli*	Selsted et al. (1992), Lee et al. (2003) and Hsu et al. (2005)
	BMAP-28	Bovine myeloid antimicrobial peptide	*C. albicans*, mammalian tumor cells	Risso et al. (2002)
	SMAP-29	Sheep myeloid antimicrobial peptide	*C. albicans*, *Pseudomonas aeruginosa*	Lee et al. (2002a), Shin et al. (2001) and Dawson and Liu (2011)
	PMAP-23	Porcine myeloid antimicrobial peptide	*C. albicans*	Park et al. (2002b) and Lee et al. (2001, 2002b)
	Protegrin-1	Porcine cathelicidin	*C. albicans*, *C. neoformans*	Dawson and Liu (2010)
	LL-37	Human secretions	*C. albicans*, Gram-positive and Gram-negative bacteria	den Hertog et al. (2005), Durr et al. (2006), and Oudhoff et al. (2010)
	HNP1, HNP-2, HNP3a	Human neutrophils	*C. albicans*, *C. neoformans*, *Coccidioides immitis*, *Rhizopus oryzae*, *A. fumigatus*	Ganz (2003, 2005), Raj and Dentino (2002), and Lehrer et al. (1988)
	Histatin 5	Human salivary peptide	*C. albicans*, *C. neoformans*, *A. fumigatus*	Xu et al. (1999), Tsai and Bobek (1997), Situ et al. (2000), and Helmerhorst et al. (2001)
	hLF(1–11), hLF (21–31)	Human lactoferrin	*C. albicans*	Lupetti et al. (2000) and Viejo-Diaz et al. (2004)

(continued)

Table 3.1 (continued)

Antifungal peptides				
Origin	Name	Species	Effective	References
Synthetic	B4010	Originated from a secondary peptide derived from human β-defensin3	*C. albicans*	Eckert (2011) and Nguyen et al. (2010)
	Penetratin 1	Cell-penetrate peptide	*C. albicans*, *C. neoformans*	Milletti (2012) and Masman et al. (2009)

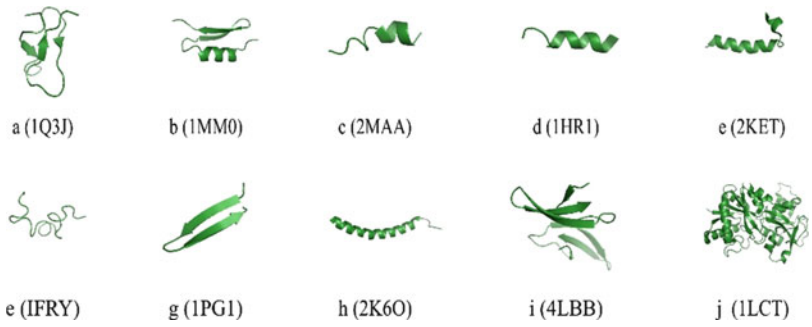

Fig. 3.2 Structures of antifungal peptides. Schematic representation of antifungal peptide based on nuclear magnetic resonance. Each PDB code is in parenthesis. Alo-3 (**a**), termicin (**b**), temporin-1 (**c**), indolicidin (**d**), BMAP-27(**e**), SMAP-29 (**f**), protegrin-1 (**g**), LL-37 (**h**), HNP1 (**i**), and (hLF) (**j**). All images were done using The Pymol molecular graphic system, v1.7.4

*De novo*peptide design includes high-throughput combinatorial library screening, structure base modeling, predictive algorithms, and introduction of non-coded modifications to conventional peptide chemistry (Nguyen et al. 2011; Blondelle and Lohner 2010). The great importance of designed peptides is that many specific properties, such as hydrophobicity, hydrophobic length, or nature of flanking residues, can be systematically varied. Ideally, artificial transmembrane peptides should serve as mimics for transmembrane segments of membrane proteins (Holt and Killian 2010). It is possible to synthetize shorter peptides structurally related to another known peptide, exploring the influence of amino acid substitutions and deletions on its antifungal activity. Linear peptides are flexible, and their possible tridimensional conformations are therefore very complex to determine. It is necessary to use tools to perform conformational analysis for these structures (Garibotto et al. 2010). Figure 3.2 shows structures of some antifungal peptides deposited in RSCB Protein Data Bank website by their authors, including its PDB code (Barbault et al. 2003; Da Silva et al. 2003; Saravanan et al. 2013; Friedrich et al. 2001; Yang et al. 2009; Tack et al. 2002; Fahrner et al. 1996; Wang 2008; Zhao et al. 2013; Day et al. 1993).

3.3 Insect Antifungal Peptides

Insect peptide Alo-3 was isolated from the coleopteran *Acrocinus longimanus*. This peptide contains six cysteine residues, forming three disulfide bridges and an antiparallel β-sheet with a long flexible loop connecting the first strand to the second strand and a series of turns. Alo-3 belongs to the knottin-type family of proteins with a cysteine-stabilized, "knotted" topology, defined by two parallel disulfide bonds, threaded by a third one. It has no negatively charged residues and displays a cationic

pole on its surface that may contribute to its antifungal activity (van der Weerden et al. 2013). Barbault and coworkers (2003) tested two other homologous peptides, Alo-1 and Alo-2, with sequence identity above 80 %, but Alo-3 was the most effective against *Candida glabrata* and *C. albicans*, both tested not only against clinical isolates of those pathogens but also against ATCC strains (ATCC90030 and ATCC 36082, respectively). Another peptide derived from insects is termicin, isolated from the fungus-growing termite *Pseudocanthotermes spiniger* (heterometabole insect, Isoptera). Termicin is a cysteine-rich antifungal peptide with a α-helical segment and two antiparallel β-sheets forming a "cysteine αβ motif," also found in antibacterial and antifungal defensins and from plants. Termicin showed activity against *C. albicans* and *C. neoformans*, but was inactive against *C. glabrata* (Da Silva et al. 2003; Lamberty et al. 2001).

Likewise, *C. albicans* growth was inhibited by holotricin-3 and tenecin-3 peptides, isolated from the hemolymph of the coleopteran insect *Holotrichia diomphalia* and from the larvae of *Tenebrio molitor*, respectively (Kim et al. 1998; Lee et al. 1995). Tenecin-3 had a better candidacidal effect, and its uptake and internalization by the cell are essential for its antimicrobial activity. This indicates an inner target involvement in the process of killing, since membrane permeabilization and calcein release were not observed. Uptake of tenecin-3 was inhibited at low temperature (0 °C) and by the presence of the oxidative phosphorylation inhibitor, sodium azide (Kim et al. 2001). Chae and coworkers (2012) isolated a new 14 kDa peptide, named tenecin-4, which was effective against *Escherichia coli* but not against *Bacillus subtilis* or *C. albicans*.

An interesting group of insect peptides is the cecropins, which were originally isolated from the cecropin moth (*Hyalophora cecropia*) and have been found in insects like *Drosophila* (van der Weerden et al. 2013). Cecropins are basic 35–39 amino acid residue peptides that can fold into two amphipathic α-helices, separated by a more flexible hinge. Their mode of action against bacteria is based on the formation of either voltage-dependent ion channels or general disruption of the membrane by a "carpet-like" mechanism (Steiner et al. 1988). Cecropin A, a 37 amino acid residue peptide, is complexed with lipopolysaccharide and in germinating cells of *Aspergillus fumigatus* induces death, whereas binding and cell death were not observed with non-germinating hyphae (De Lucca et al. 1997). De Lucca and coworkers (2000) have proposed the mode of action of this peptide as involving disruption of the plasma membrane. The ABP-dHC-cecropin A (antimicrobial peptide drury *Hyphantria cunea*), a highly cationic peptide isolated from the fat bodies of drury moths (*H. cunea*), has shown a strong antifungal activity against both *C. albicans* and *Neurospora crassa* as well as *Rhyzopus*, *Fusarium*, *Alternaria*, and *Mucor* species (Zhang et al. 2015).

Riciluca and coworkers (2012) have recently isolated a peptide named rondonin from the spider *Acanthoscurria rondoniae*. This peptide shows a molecular mass of 1236.77 Da and activity against *C. albicans*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii*. Otherwise, no deleterious activities against human erythrocytes or Gram-positive and Gram-negative strains were observed.

3.4 Amphibian Antifungal Peptides

Amphibians inhabit environments that provide a great challenge for their immunity, providing valuable information about prospective functional molecules. In this context, peptides have been isolated and described. Some of them, like brevinins and temporins, were effective against human fungal pathogens (Xu and Lai 2015).

Brevinins consist of two families named brevinin-1 (24 residues) and brevinin-2 (33–34 residues), and they were first described in 1992 by Morikawa and coworkers (Morikawa et al. 1992), who isolated these peptides from the skin of the Japanese frog *Rana brevipoda*

porsa, demonstrating microbicidal activity against a wide range of Gram-positive, Gram-negative bacteria and pathogenic fungi strains. Up to now about 350 types of brevinins have been discovered, sharing common features like linearity, amphipathicity, and cationicity, and some of them have a C-terminal disulfide-bridge cyclic heptapeptide, called a rana box (Novkovic et al. 2012; Savelyeva et al. 2014). Brevinin-1 exists as a random coil in aqueous solution, but adopts an amphipathic α-helical structure in a hydrophobic membrane-mimetic environment such as 50 % trifluoroethanol. The brevinin-1 peptides generally comprise an N-terminal hydrophobic region, a proline containing a hinge region in the central portion, and a C-terminal disulfide-bonded loop (Kwon et al. 1998). The α-helical structure leads to perturbation of the phospholipid bilayer of target membranes in the "barrel-stave" and "carpet-like" models (Savelyeva et al. 2014)

Conlon and coworkers (2003) isolated the peptide brevinin-1BYa (FLPILASLAAKFGPKLFCL VTKKC) from the norepinephrine-stimulated skin secretions from the foothill yellow-legged frog *Rana boylii*. This peptide was potent against *C. albicans* and *Staphylococcus aureus*, but its therapeutic potential is limited due to its strong hemolytic activity. The research group has also substituted amino acid in the original molecule, which leads to brevenin-1BYb and brevenin-1BYc peptides with fourfold and tenfold reduction against *C. albicans*, respectively. The change in the cationic residues can be the explanation of the observed result, since this global net charge reduction affects the initial binding to the negatively charged phospholipids in the microorganisms' cell membranes (Yeaman and Yount 2003). Another study evaluated the antimicrobial activity of brevinin-1BYa and investigated the growth inhibitory activity of a synthetic replicate of this peptide: [Ser^{18}, Ser^{24}] brevinin-1BYa (FLPILASL AAKFGPKLFSLVTKKS). The group observed an eightfold reduced hemolytic activity compared to the native peptide and suggested that this reduction arises from destabilization of the α-helix in the C-terminal region of the peptide associated with replacement of the cysteine bridge. Antimicrobial activities against *C. albicans* and Gram-negative bacteria were reduced. In contrast, substituting the two cysteines for serines abolished the antifungal activity (Park et al. 2002a; Pal et al. 2006). Peptides isolated from *Rana pipiens*, such as brevinin-1Pa, brevinin-1Pb, and brevinin-1Pc, were also effective against *C. albicans*, *S. aureus*, and *Escherichia coli* (Goraya et al. 2000). In addition, brevinins are able to stimulate insulin release, which causes hypoglycemia in frogs attacking predators. This property can also be explored for the treatment of patients with type 2 diabetes (Marenah et al. 2004; Abdel-Wahab et al. 2010).

Another class of amphibian peptides is the temporins. They were initially identified in 1996 in skin secretion of the European red frog *Rana temporaria* (Simmaco et al. 1996), but they can be isolated from several other frog species as well as from wasp venom (Rollins-Smith et al. 2003). They are a group of linear short peptides (10–14 amino acid residues) with a net cationic charge and an amidated C-terminus. In an apolar environment, temporins showed a marked propensity to adopt an amphipathic α-helical structure. Temporins are not as basic as other cationic peptides, although in the most potent, temporin A, the one basic residue is essential for activity. Temporins A and B have been reported to be active against *C. albicans* and Gram-positive and Gram-negative bacteria. These peptides permeate both artificial and biological membranes, but they do not lyse human erythrocytes, which suggests there are additional factors involved in the mechanism of action on different cell types (van der Weerden et al. 2013; Mangoni et al. 2000; Wade et al. 2000; Hujakka et al. 2001; Carotenuto et al. 2008).

3.5 Mammalian Antifungal Cathelicidins

Cathelicidins are peptides of approximately 100 amino acid residues, and their sequences are related to cathelin, a cystatin-like protein. They are commonly found in humans and other species such as sheep, pigs, horses, cattle,

chickens, rabbits, and some species of fishes, being usually stored in the secretory granules of neutrophils and macrophages. They can also be released extracellularly upon leukocyte activation (Zanetti 2005; Kosciuczuk et al. 2012). The term cathelicidins was first proposed in 1995 to acknowledge the evolutionary relationship of the novel protein family to cathelin, and it is used to denote holoproteins that contain a cathelin-like sequence and a cationic antimicrobial domain (Zanetti et al. 1995). The first is a conserved N-terminal sequence ("cathelin" domain, the cathepsin L inhibitor), and the second is a C-terminal antimicrobial domain of varied sequence and length (both interspecies and intraspecies), which express their activity after they have been cleaved from the holoprotein (Gennaro and Zanetti 2000). In mammals, cathelicidins were first identified in bone marrow myeloid cell, and therefore they are also named "myeloid antimicrobial peptides" (MAP) (Zanetti 2005).

Among the cathelicidin peptides, some present antifungal activity and this is stronger against yeast than against filamentous fungi (Benincasa et al. 2006). Indolicidin is a tryptophan-rich bovine cathelicidin peptide of 13 amino acid residues (ILPWKWPWWPWRR-NH2), purified from the cytoplasmic granules of neutrophils and found in bone marrow cells as a 144-long amino acid precursor (Selsted et al. 1992; Del Sal et al. 1992). This peptide showed activity against fungi *C. albicans* and *C. neoformans*, as well toward bacteria *S. aureus* and *E. coli* (Benincasa et al. 2006). The fungicidal activity involves membrane disruption, DNA binding and topoisomerase 1 inhibition. In the first situation, the peptide interacts with the lipid bilayers in a salt- and energy-dependent manner. The DNA interaction occurs by DNA synthesis inhibitors binding DNA or proteins involved in the process. Indolicidins may also interact in other biosynthesis pathways or in cell cycle signal transduction (Lee et al. 2003; Hsu et al. 2005).

Other bovine cathelicidins with fungicidal activity are bovine myeloid antimicrobial peptides (BMAP) of 27 and 38 amino acid residues, BMAP-27 and BMAP-28, respectively. BMAP-28 is toxic for mammalian tumor cells, inducing their apoptosis, and it was also demonstrated that it induces mitochondrial permeability, forming transition pores (MPTP), resulting in the release of cytochrome *c*. The cytotoxic activity has been related to the structural features of the peptide, which consists of a cationic N-terminal sequence predicted to assume an amphipathic α-helical conformation (residues 1–18) and a C-terminal hydrophobic tail (residues 19–27). This hydrophobic tail is responsible for the peptide activity, since its analogue, BMAP28 (1–18), which comprises the 18 N-terminal residues, showed a reduction in MPTP effect. BMAP28 cytotoxicity requires an active metabolism of the target cells (Risso et al. 2002).

SMAP-29 is cathelicidin-like peptide derived from myeloid sheep with α-helical structure in a hydrophobic environment, and its C-terminal hydrophobic domain has a strong membrane permeability (Chen et al. 2011; Skerlavaj et al. 1999). This peptide concentrates on the plasma membrane of treated cells and causes propidium iodide (PI) uptake, provided by the cells that are metabolically active. Lee and coworkers (2002a) suggest that membrane disruption by SMAP-29 occurs via pore formation, due to a direct interaction with the lipid bilayers and irregularly disrupted fungal membranes in an energy- and salt-dependent manner. SMAP-29, however, is strongly hemolytic against human erythrocytes. A variant of SMAP-29, [$K^{22,25,27}$]-SMAP-29, is effective against bacterial and fungal cells in physiological salt concentrations and was not injurious to eukaryotic cells, such as human erythrocytes (Shin et al. 2001; Dawson and Liu 2011).

Isolated from porcine myeloid, peptide PMAP-23 was identified by cDNA cloning and is 23 residues long, cationic (+7), and amphipathic. In a hydrophobic environment, it forms two α-helices joined by a flexible region when membrane-bound (Roversi et al. 2014; Park et al. 2002b). This peptide is capable of binding to plasma membrane of *C. albicans* protoplasts, indicating that an interaction with the cell wall is not a requirement for the inhibitory activity of

this peptide, which also did not show hemolytic activity (Park et al. 2002b; Lee et al. 2001). Lee and coworkers (2002b) designed several analogs of PMAP-23, with amino acid substitutions in order to increase the net hydrophobicity by Trp (W)-substitution at positions 10, 13, or 14 on the hydrophilic face of the peptide. In *C. albicans* the P6 analog peptide exerted its fungicidal effect on the blastoconidia by disrupting the mycelial forms, causing significant morphological changes. Meanwhile, P6 also displayed about fourfold greater antitumor activity than the parent PMAP-23.

Porcine cathelicidins, such as protegrin-1 (PG-1), showed activity against clinical isolates of fungi, including those resistant to conventional medicines used in human therapy (Benincasa et al. 2006). These peptides and their variants have a rather rigid antiparallel β-sheet (β-hairpin) structure that is stabilized by two intramolecular disulfide bonds. The linear peptide forms have been reported to be considerably less active than the native form, being sensitive to physiological salt concentrations (Dawson and Liu 2010). PG-1, BMAP-27, BMAP-28, SMAP-29, and indolicidin showed deleterious activity against a number of nosocomial yeast strains, mainly *Candida* spp. and *C. neoformans* (Benincasa et al. 2006).

The most famous prototype of cathelicidin group is human cathelicidin LL-37, which attains a helical structure when bounded to cell wall and plasma membrane of treated cells and has the protein hCAP-18 as its precursor. LL-37 is secreted in human sweat and further processed into RK-31 and KS-30, more active peptides that retain their activity even in high salt conditions. Moreover, they are able to enter the cytoplasm of *C. albicans* cells, suggesting that their increased activity may result from interaction with intracellular targets. In contrast, LL-37 could not be detected in the cytoplasm (den Hertog et al. 2005, 2006; Lopez-Garcia et al. 2005). LL-37 showed a pH-dependent activity against *C. albicans*, disrupting its membrane and allowing leakage of proteins of up to 40 kDa into the medium (den Hertog et al. 2005). This peptide discriminates against phospholipid monolayers containing negatively charged lipids, as SMPA-29 does. However, experiments comparing these two peptides demonstrated that the LL-37 peptide had a more potent effect than the SMAP-29, suggesting that its interaction with monolayers involves other factors, such as hydrophobicity, size, and charge distribution, although SMAP-29 has a higher net positive charge (+10), and it would be expected to be more attracted to the negatively charged lipid monolayers (Neville et al. 2010). When compared to histatin-5, LL-37 induced higher morphological defects, but the efflux of nucleotides is similar in comparable candidacidal concentrations, suggesting that the loss of nucleotides plays an important role in the killing process (den Hertog et al. 2005). LL-37 has been found to have additional activities, such as regulating the inflammatory response and chemo-attracting cells of the adaptive immune system to wound or infection sites, helping to neutralize the microorganism and promoting re-epithelization and wound closure (Durr et al. 2006; Oudhoff et al. 2010).

3.6 Mammalian Antifungal Defensins

Mammalian defensins are a large group of peptides with an important role in the host's immune system. They can be divided into the α- and β-defensins based on their structural characteristics and cysteine spacing pattern (van der Weerden et al. 2013). Both defensins were first identified as antimicrobial compounds involved in innate immunity. In humans, α- and β-defensins are expressed mainly in different sites: the α-defensins are mostly expressed in neutrophils (known as human neutrophil peptides (HNP), or human defensins (HP) when expressed in natural killer cells) and the β-defensins are secreted by the epithelial cells of the skin and mucosae and known as HβD (Suarez-Carmona et al. 2014). Apart from the antimicrobial activities, the defensins appeared as modulators of the adaptive immune system and angiogenesis, key mediators of wound healing and

determinant players in male fertility (Ganz 2003; Oppenheim et al. 2003). The α-defensins comprise 29–35 amino acid peptides that share six conserved cysteine residues with three disulfide bonds. Their structure is formed by an amphipathic and antiparallel β-sheet. They also have a β-hairpin loop containing cationic charged molecules. The β-defensins are longer than their α-counterparts (34–42 residues in length) and are triple-stranded with an antiparallel β-sheet as well as a short α-helix (De Smet and Contreras 2005; Selsted and Ouellette 2005). The human defensins present activity against a wide range of microorganisms, including fungal pathogens. HNP 1–3 are identical apart from one N-terminal amino acid, which makes HNP 3 completely inactive against *C. albicans*, while HNP 1–2 is candidacidal. HNP 4 is also toxic to *C. albicans* cells, and the mechanism of action of those peptides on fungal cells has been proposed to be by membrane permeabilization (Ganz and Lehrer 1995; Ganz 2005). NP 1 seems to work in the same way, but differently from HNP, and it was shown to be dependent on the metabolic activity of the target cells. HNP-1 causes *C. albicans* to release ATP, just as histatin 5 does, but in contrast, it did not seem to lyse cells. NP-1, NP2, and NP3a were highly effective against *C. albicans*, and NP-1 was effective against *C. neoformans*, *Coccidioides immitis*, and hyphae and germinating conidia of *Rhizopus oryzae* and *Aspergillus fumigatus* (van der Weerden et al. 2013; Raj and Dentino 2002; Lehrer et al. 1988).

Moreover, β-defensins, HβD 2 and HβD 3, are potent inhibitors of *C. albicans*. Exposure to this microorganism and to *Trichophyton rubrum* and *A. fumigatus* stimulates HβD 2 expression. *A. fumigatus* also induces the expression of HβD 9. The mechanisms of action of these peptides are not well known, but some requirements seem to be necessary for their activities, such as metabolically active cells and a low concentration, since they have a strong positive net charge, which in high concentrations of cations may decrease efficacy (Dhople et al. 2006; Joly et al. 2004; Liu et al. 2002; Alekseeva et al. 2009). Another antifungal peptide is Novexatin, a cyclic and highly cationic peptide (1,093 daltons), arginine rich, based on the human α and β defensins. It is currently in phase 1/2 of clinical trials for use against fungal infections of the toenails; NovaBiotics (Aberdeen, UK) is the company responsible for its development (Fox 2013).

3.7 Mammalian Antifungal Histatins

The histatin family consists of 12 members of histidine-rich peptides from which histatins 1 and 3 (the full-length proteins and gene products) and histatin 5 (a cleavage product of histatin 3) are the main ones and constitute 70–80 % of the total amount (Xu et al. 1999). The other nine members are proteolytic cleavage products of these peptides (Fitzgerald et al. 2003). Although they are named due to a high number of histidine residues, other amino acids including lysines (Lys^5 and Lys^{13}) rather than histidines have key importance for fungicidal activity (Kumar et al. 2011; Rothstein et al. 2001). Studies demonstrate that histatins have a number of biological activities in vitro, such as the maintenance of tooth surface integrity, histamine release induction, and potentiation of rabbit chondrocyte growth (Hay 1975; Oppenheim et al. 1986; Sugiyama et al. 1985; Murakami et al. 1994). The histatins are encoded by two closely related genes (HIS1 and HIS2), with histatin 1 and histatin 3 as primary products of HISI and HIS2, respectively (Sabatini and Azen 1989).

Histatin 5 was obtained from histatin 3 (Raj et al. 1998) and has the strongest antimicrobial activity against pathogenic fungi *C. albicans*, *C. neoformans*, and *A. fumigatus* (Kavanagh and Dowd 2004). This histatin is 24-amino acid residues long with seven histidines, four arginines, and three lysines, and its fungicidal activity resides in a region of 11–24 residues at the C-terminal, referred to as the functional domain or dh-5 (Driscoll et al. 1995). In a non-aqueous environment, the peptide adopts a α-helical conformation, and, like histatin 3, in

an aqueous environment it adopts a random coil structure (van der Weerden et al. 2013; Helmerhorst et al. 1999a; Tsai and Bobek 1997). The ability to form a α-helix was thought to be important for the mode of action of histatin 5, but Situ and coworkers (2000) demonstrated that one variant of histatin 5 (3P) with reduced ability to form α-helix had an antifungal ability comparable to that of histatin 5. Its antifungal mechanisms against *C. albicans* involve binding to a specific receptor, translocation across the membrane, and interaction with internal targets such as mitochondria and non-lytic release of cellular ATP (Fitzgerald et al. 2003; Helmerhorst et al. 1999b; Koshlukova et al. 1999, 2000). Histatins do not display lytic activities to lipid membranes, measured by release and dequenching of the fluorescent dye calcein (Edgerton et al. 1998).

Histatin interaction with the cell wall and its uptake by the cell are two independent events, since the fungal cell wall binding itself does not result in uptake of histidines. Li and coworkers (2006) demonstrated that the heat shock protein Ssa2p is the binding site for histatin 5 measured by yeast two-hybrid analyses, whereas Ssa1p appears to have a lesser role in histatin 5 toxicity. This heat shock protein 70 (Ssap 1/2) is located in the fungal cell envelope. These interactions, however, can be prevented in the presence of Ca^{+}, which presumably disrupts the interaction between histatin 5 and Ssa2p (Li et al. 2006). Internalization of histatin 5 occurs by translocation, and its uptake is dependent on two polyamine transporters, Dur3 and Dur31 (which usually function in spermidine uptake), since *C. albicans* showed a reduced intracellular transport of histatin 5 upon growth in a medium rich in spermidine, implicating polyamine transporters in uptake of this peptide (van der Weerden et al. 2013; Kumar et al. 2011).

The mitochondrion's primeval functionality is as an energy-generating organelle, although the molecular machinery in charge of its role shows a broad divergence among different phyla (Tielens et al. 2002). It has been proposed that a negatively charged phospholipid in the mitochondrion membrane, called cardiolipin, attracts histatin 5 toward the mitochondrion. This interaction causes ATP release into the cytoplasm, and it was shown by the evidence of unchanged levels of ATP when cells were treated with respiration uncouplers, such as azide or cyanide, and then exposed to histatin 5, indicating that respiration is essential for histatin activity to occur (Helmerhorst et al. 1999a; Koshlukova et al. 1999). Gyurko and coworkers (2000) also demonstrated that fungal cells incapable of respiration (respiratory-deficient or petite mutants) are resistant to the action of histatins. The ATP efflux occurs via ATP-binding cassette (ABC) proteins, and extracellular ATP activates a purigenic-like receptor that triggers cell death (Koshlukova et al. 2000). Another ATP release consequence is the effect on the regulation of cell volume homeostasis, which can halt cells at the G1 phase, disrupting the cell cycle and leading to cell death that is not in a programmed pathway (Wunder et al. 2004). Histatin 5 is responsible for the generation of reactive oxygen species (ROS), which is measured using an oxygen radical sensitive probe (dihydroethidium), and it is one of the components responsible for the disruption of cell organelle structure or function (Baev et al. 2001). Another important factor of histatin-5 is that it retards the transition of *C. albicans* from the blastopore to the hyphal stage of growth, a process that may assist in arresting tissue penetration by the fungus (Helmerhorst et al. 1997).

3.8 Mammalian Lactoferrin-Derived Antifungal Peptides

Lactoferrin (Lf) is a multifunctional protein (80 kDa), a member of the transferrin family of non-heme iron-binding glycoproteins that is expressed and secreted by granular epithelial cells and secreted into mucosal fluids that bathe the body surface; it is found in the secondary granules of neutrophils during the myelocyte stage of maturation (Ward et al. 2005; Levay and Viljoen 1995). It was first isolated from bovine milk and later identified in mice, pigs, and humans (van der Weerden et al. 2013). On the mucosal surface, this peptide works as a

component of the first line of host defense, being released from neutrophils during infection, inflammation, tumor development, and iron overload (Levay and Viljoen 1995; Legrand et al. 2008). It also acts in the regulation of iron homeostasis, cellular growth, and differentiation and protection against cancer development and metastasis (Ward et al. 2005; Shimamura et al. 2004).

Proteolytic cleavages of lactoferrin revealed some peptides (lactoferricins) with better antifungal activity than that of the whole protein, such as the first and second cationic domains derived from human lactoferrin (hLF) hLF (1–11) and hLF (21–31), respectively (Lupetti et al. 2000). A study using those synthetic peptides revealed a dose-dependent release of ATP by *C. albicans* upon exposure to hLF (1–11). The same study demonstrated that a metabolic active cell is necessary for the hLF (1–11) mode of action, since cells incubated with sodium azide had a reduced candidacidal activity and a lower PI uptake. The use of the fluorescent dye rhodamine 123 showed an accumulation inside the mitochondria and later was released into the cytoplasm when cells were treated with hLF (1–11), which indicates that the peptide triggers the energized mitochondrion (Lupetti et al. 2000). This ATP efflux was also observed in a short synthetic peptide following the N-terminal amino sequence of bovine lactoferrin (peptide 2 or Pep2). Pep 2 activated pertussis toxin-insensitive and cholera toxin-sensitive G-protein and activated signals downstream through phosphatidylinositol 3-kinase to protein kinase C. This indicates that Pep 2 induced ATP efflux mediated by G-protein activation (Tanida et al. 2006; Helmerhorst et al. 2002). Another study showed that cell wall interaction and therefore membrane binding with hLF are not the major mode of action, thus demonstrating a slight efflux of K^+ from *C. albicans* cells, but this did not allow Na^+ release or membrane disruption (Viejo-Diaz et al. 2004). Some other peptides, derived both from bovines (LfcinB), which comprise the region spanning 17–40 residues, and from humans (LfcinH), dissipated the proton gradient across the plasma membrane. However, they did not seem to act by nonspecific permeabilization of the membrane as they did not cause calcein release from artificial liposomes (Nguyen et al. 2005; Viejo-Diaz et al. 2003).

3.9 Synthetic Antifungal Peptides

Natural peptides can have their activity enhanced by peptide engineering, which can help to develop novel peptides with desirable biological properties (Ryu et al. 2014). The synthetic peptide B4010 originated from a 10-residue peptide (RGRKVVRRKK), which in turn is a synthetic analogue of human β-defensin-3 (HβD-3). Its properties have been previously reported by Lakshminarayanan and coworkers (2014) as showing potent activity against *Pseudomonas aeruginosa*, but poor activity against fungi (Bai et al. 2009).

That research group also extended the previous analysis to a higher order of covalently linked peptides. B4010 is a tetravalent synthetic peptide, which carries four copies of the sequence RGRKVVRR through a branched lysine core. This strategy of developing multivalent peptides by assembling multiple copies of monomeric peptides around a core molecule is an alternative to circumventing the drawbacks of antimicrobial peptides, such as the loss of antimicrobial properties in a physiological concentration of salts and polyanionic polymers (Knappe et al. 2010; Eckert 2011).

B4010 presented deleterious activity against *C. albicans* strains (Lakshminarayanan et al. 2014). The researchers also tested another peptide by linking two copies of the sequence through a branched lysine, B2088, and observed a substantial decrease in MIC (minimal inhibitory concentration) values when compared to the monomer or linear retrodimer peptide (RGRKVVRRKKRRVVVKRGR). The MIC values of B4010 (0.37 μM) for two clinical isolates of *C. albicans* were also lower when compared to the MIC values for amphotericin B (1.4 μM) and natamycin (15 μM). This last one is the only US FDA-approved antifungal for ophthalmic applications (Lakshminarayanan

et al. 2014; Arora et al. 2011), which is also nonhemolytic and nontoxic to mice when administrated by intravenous (100 $mg.kg^{-1}$) or intraperitoneal (200 $mg.kg^{-1}$) routes and had no affinity for cell wall polysaccharides. It was proposed that its mode of action includes a rapid dissipation of membrane potential and release of vital ions and ATP when challenged by *C. albicans*, and some studies suggest that the first arginine is important for mediating peptide-bilayer interactions (Lakshminarayanan et al. 2014; Nguyen et al. 2010).

Cell-penetrating peptides (CPPs) are part of a group of synthetic peptides with up to 30 peptides and are able to enter cells in an energy-independent manner, translocating across the membrane (Milletti 2012). Penetratin 1 is a 16 amino acid long CPP from the third helix of the Antennapedia homeodomain of *Drosophila*. It can be classified as a cationic amphipathic peptide, and its proposed mechanism of action is by "inverted micelle" pathway. This peptide was synthetized and had its fungicidal activity evaluated by Masman and coworkers (2009). This research group tested not only penetratin 1 against *C. albicans* and *C. neoformans* but also tested other peptide sequences, among them a trapeptide (RQKK-NH_2), identified as 8 in their study. Both peptides, 1 and 8, displayed the most potent inhibitory effect against those pathogenic fungi (Garibotto et al. 2010; Masman et al. 2009).

3.10 Prospects for Clinical Use of Antimicrobial Peptides

The development of new drugs is a remarkable challenge. The last new class of antibiotics, the lipopeptide daptomycin, was introduced in 2003, more than 40 years after the introduction of fluoroquinolones, the last antibiotics used to treat multidrug-resistant (MDR) organisms such as *Klebsiella* and *Acinetobacter* species. Peptides are a class of molecules with potential for therapeutic use against fungal infections (Steckbeck et al. 2014), but their use as novel drugs has to overcome some therapeutic difficulties, such as their poor chemical and physical stability and short circulating plasma half-life. Additionally, those molecules are antagonized by physiological concentrations of salt and polyanionic polymers (mucins, DNA, and glycosaminoglycans) and by the action of proteolytic enzymes, thus limiting their therapeutic potential (Lakshminarayanan et al. 2014; Knappe et al. 2010; Otvos and Wade 2014). Another difficulty is the expensive manufacturing of peptides when compared to small-molecule drugs. Companies are scrambling for financial support both from federal programs and from corporate partners through the later and most expensive stages of clinical evaluations (Fox 2013).

AMPs have only been tested in clinical trials relatively recently, and to date, with the exception of gramicidin for topical administrations, none has received US Food and Drug Administration (FDA) approval. Kaspar and Reichert (Kaspar and Reichert 2013) highlight that in 2012 the first marketing approvals were made for six peptides (lucinactant, peginesatide, pasireotide, carfilzomib, linaclotide, and teduglutide) in the United States and European Union, which only disapproved peginesatide due to safety issues. Another example of a peptide that was undergoing clinical trials is pexiganan, a synthetic analog of the AMP magainin. Pexiganan had impressive results in early phase I and II clinical trials to treat diabetic ulcers, but its performance was not superior when compared to traditional antibiotics used in treating feet ulcers (Moore 2003). At present, approximately 140 peptide therapeutics are being evaluated in clinical trials (Fosgerau and Hoffmann 2015).

In spite of all the challenges, antimicrobial peptides have the potential for development as novel antifungal agents, because they have rapid action and a broad spectrum, being active against species that are multiple resistant to currently used antimycotics. Moreover, peptides are selective and efficacious signaling molecules with specific targets, properties that are important features for drug safety, and they show diminished side effects for the patient (Matsuzaki

2009; Wang et al. 2010; Takahashi et al. 2010). Natural or designed peptides can avoid cell toxicity and hemolytic activity and other undesirable features (Fjell et al. 2012). Some chemical strategies that have been used are sugar molecules incorporated in the peptide N-terminus, which may improve tissue penetration, and the conjugation to passive and active transport enhancers in order to increase oral bioavailability (Charlton et al. 2008; Gupta et al. 2013; Sachdeva et al. 2013). In addition, peptides play important roles in innate immune response and wound healing, additional features for their antimicrobial activity (Pena et al. 2013; Steinstraesser et al. 2012).

In summary, peptides are promising molecules with potential as future therapeutics for fungal diseases. Research is imperative to understand their mode of action and specific targets against fungi and to improve their pharmacological properties to meet clinical therapeutics and drug manufacturing needs.

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