

Alternative approaches to antifungal therapies

Tarun Mehra, Martin Köberle, Christina Braunsdorf, Daniela Mailänder-Sanchez, Claudia Borelli and Martin Schaller

Department of Dermatology, Eberhard-Karls-University, Tübingen, Germany

Correspondence: Martin Schaller, Department of Dermatology, University Hospital of Tübingen, Liebermeisterstraße 25, 72076 Tübingen, Germany, Tel.: 0049 7071 2984555, Fax: 0049 7071 29 4405, e-mail: martin.schaller@med.uni-tuebingen.de

Abstract: The expansive use of immunosuppressive medications in fields such as transplantational medicine and oncology, the higher frequency of invasive procedures in an ageing population and the HIV/AIDS pandemic have increased the frequency of systemic fungal infections. At the same time, increased resistance of pathogenic fungi to classical antifungal agents has led to sustained research efforts targeting alternative antifungal strategies. In this review, we focus on two promising approaches: cationic peptides and the targeting of fungal virulence factors. Cationic peptides are small, predominantly positively charged protein fragments that

exert direct and indirect antifungal activities, one mechanism of action being the permeabilization of the fungal membrane. They include lysozyme, defensins and cathelicidins as well as novel synthetic peptides. Among fungal virulence factors, the targeting of candidal secreted aspartic proteinases seems to be a particularly promising approach.

Key words: antifungal therapies – antimicrobial peptides – cathelicidin – defensin – inhibitors – Sap

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Facultative pathogenic fungal organisms such as *Candida albicans* colonize mucosal surfaces of the majority of humans, without provoking a clinical infection in immunocompetent individuals (1,2). The role of antimicrobial peptides in the prevention of fungal infections has recently received increased attention. These small, predominantly cationic proteins are key elements of innate immunity and can directly kill multiple bacterial, viral and fungal pathogens. Far from being solely restricted to an effector role, they also exert their antimicrobial activity as immunomodulating agents (3) or inhibit virulence factors of the pathogen. They are found on the epidermis as well as the mucosa and form a soluble barrier against a pathogen invasion, acting jointly with an intact epidermis as a first line of defence. Another attractive approach is the use of inhibitors of fungal virulence factors such as candidal aspartic proteinases. We review alternative approaches to antifungal treatment with a focus on antifungal peptides and proteinase inhibitors. An overview is given in Table 1.

Antifungal peptides

RNase-7

Discovered in 2002 by J Harder and JM Schröder, this 14.5-kDa ribonuclease exerts broad antimicrobial activity against *C. albicans* as well as numerous Gram-positive and Gram-negative bacteria, especially *Enterococcus faecium*. Crude extracts of callus stratum corneum of healthy individuals showed antimicrobial activity. Analysis with high-performance liquid chromatography demonstrated a fraction with a high activity against *Staphylococcus aureus*. This fraction was further purified and analysed with mass spectrometry, showing a 14.5-kDa protein, which was sequenced with Edman degradation. The isolation of the corresponding cDNA from primary keratinocytes enabled the deduction of the complete amino acid sequence that showed the greatest similarity to RNase A superfamily members. *In vitro* antimicrobial activity was quantified by counting colony-forming units (CFUs) of microbes cultivated with increasing amounts of RNase-7. A mid-range dose of 4 μM achieved a CFU reduction in *C. albicans* from

10^5 to a little more than 10^3 on a logarithmic scale. Analysis of tissue from various body regions revealed an RNase-7 expression in numerous epithelial tissues, such as skin, genitourinary tract and respiratory tract as well as at low levels in the digestive tract. RNase-7 was also detected in the supernatant of the culture of unstimulated primary keratinocytes. Real-time PCR showed an upregulation of RNase-7 expression in primary cultured keratinocytes after treatment with the proinflammatory cytokines TNF- α (2.5-fold), Interferon- γ (sevenfold) and Interferon-1 β (8.5-fold), as well as after stimulation with heat-inactivated bacteria such as *Pseudomonas aeruginosa* (ninefold) *in vitro*. The antifungal mechanisms of action so far remain unknown (4).

Lysozyme

Lysozyme is an enzyme classically known for its muramidase activity lysing bacterial peptidoglycan and killing bacteria. However, as early as 1970, it was shown that lysozyme was also active against *C. albicans*, its mechanism of antifungal action staying subject to speculation (5). Subsequently, broad antifungal activity was also shown against numerous clinical isolates of all *Candida spp.*, with a significant variation of interstrain and intrastrain sensitivity and against *Aspergillus fumigatus* and *Penicillium spp.* (6).

Lysozyme is found in virtually all human body fluids (e.g. saliva, respiratory secretions and liquor). Expression of lysozyme in the skin has been located in the cytoplasm of epidermal cells and throughout the pilosebaceous apparatus (6), in body secretions such as saliva and in neutrophils (7). Wu et al. investigated the effect of lysozyme on the viability and Sap activity of *C. albicans*. Saps are secreted aspartic proteases, and their main functions are probably to provide nutrition, aid penetration and invasion and evade immune responses (8). An incubation period of 24 h with sublethal concentrations of lysozyme resulted in a dose-dependent reduction in Sap activity and secretion, measured by spectrophotometry and ELISA, respectively. This finding was paralleled by decreasing *Candida* viability at higher, lethal concentrations (15 and 20 $\mu\text{g/ml}$), with significant differences in various strains,

quantified by colony-forming units yielded per ml cultivated medium. Electron microscopy showed ballooned cells, some appearing collapsed and deflated, after 24-h exposure to lysozyme at concentrations below 10 $\mu\text{g/ml}$, which did not affect the viability of *C. albicans*. The fungicidal activity at high concentrations therefore was suggested to result in membrane or cell wall damage leading to osmotic imbalance (9). Also, the negatively charged domain of lysozyme has been assumed to target the *Candida* surface (9). Very recently, it could be demonstrated that the digestion of human milk lysozyme by pepsin yields five different antimicrobial peptides that are also active against *C. albicans* (10).

Chitotriosidase

Besides lysozyme, other chitin-cleaving enzymes have been found in human leucocytes (11). Activated human macrophages as well as neutrophils have been demonstrated to secrete chitotriosidase, an enzyme that is implied in the defence of fungal pathogens and is also a marker for Gaucher's Disease, a lysosomal storage disorder (12). Recently, gene therapy with chitotriosidase-transfected phagocytes has been suggested as a novel therapeutic approach to combat fungal infections (13). Although it seems an interesting target for the future development of antifungal therapies, it is not expressed either in skin or mucosa.

Lactoferrin

Lactoferrin is an iron-binding protease present in various body secretions, including saliva. Human lactoferrin has found to be effective against *C. krusei* and *C. albicans* (14). It has been originally thought that lactoferrin restricts microbial growth by iron depletion. However, additional mechanisms of antimicrobial action have been reported since that time. Most notably, like with lysozyme, peptic digestion yields a cationic antimicrobial peptide (lactoferricin) with a very broad microbicidal activity (15)

Antileukoprotease (ALP, SLPI)

Antileukoprotease, a serine protease inhibitor is found in secreted fluids from the genitourinary and respiratory tract, as well as in keratinocytes from the skin and the mucosa. Tomee et al. (16) investigated the antifungal activity of recombinant ALP against *A. fumigatus* and *C. albicans*. The fungicidal activity was measured in the percentage reduction in CFUs on agar plates after incubation of *A. fumigatus* conidia and *C. albicans* yeast cells with rALP. Recombinant ALP had a dose-dependant fungicidal effect on metabolically active *A. fumigatus* conidia, while dormant *A. fumigatus* conidia were not affected. The N-terminal domain had a higher fungicidal activity in comparison with the C-terminal domain, in all concentrations tested. Lysozyme and human neutrophil defensins 1–3 showed similar fungicidal activity. Recombinant ALP had a dose-dependant effect on *C. albicans* yeast cells. It also had a dose-dependent, fungistatic effect on *C. albicans*, delaying the onset of growth of remaining colonies and increasing the lag phase of candidal growth in treated colonies. Lysozyme and human neutrophil defensins 1–3 showed similar maximum fungicidal activity, although only lysozyme showed effects at low doses. ALP also has an antiretroviral activity against HIV-1 and is bactericidal towards *Escherichia coli* and *S. aureus* (16).

Calprotectin (Calgranulin)

Calprotectin is a heterodimer constituted by S100A8 and S100A9, two low-weight proteins with two calcium-binding helix-loop-helix structural domain each. It is distributed unequally over the human skin, with barely detectable levels in the epidermis of the abdomen and high levels in the epithelial tissue of the female

genital area and of plantar skin (17). It is fungistatic towards *C. albicans*, possibly via sequestration of Zn^{2+} or Mn^{2+} . Murthy et al. (18) showed that Calprotectin inhibited candidal growth at concentrations equal to or greater than 18 $\mu\text{g/ml}$, by two orders of magnitude. Abtin et al. have shown that IL-1 α and flagellin, a protein found on some Gram-negative bacteria, induced the expression of S100A8 and S100A9 as well as *S100A8* and *S100A9* transcription. Knock-down with TLR5-specific siRNA inhibited the increase in *S100A8* and *S100A9* expression, showing that the induction of S100A8 and S100A9 by flagellin requires the TLR5-mediated pathway. Culture supernatants of *C. albicans* could not increase S100A8 and S100A9 expression (17).

Histatins

Histatin-1 and Histatin-3 are salivary proteins secreted by the parotid and submandibular glands. Histatin-5 is a 24 amino acid long N-terminal fragment of Histatin 3 and is generated by proteolytic cleavage. Histatin-5 has the strongest fungicidal activity, killing yeast and filamentous forms of *Candida* species at physiological concentrations of 15–30 μM . It exerts its fungicidal activity by binding to a candidal 67-kDa Hst-5-binding protein and subsequent non-lytic ATP efflux (19).

Defensins

Defensins are a family of small cationic peptides with six cysteine residues that can be divided into α -, β - and θ -defensins according to the alignment of their disulphide bridges and molecular structure (3). Humans only express α - and β -defensins (20). Here, we would like to focus on β -defensins.

Human β -defensin-1 is constitutively expressed in all endothelial tissues and its mRNA can be upregulated via TLR-mediated stimulation through pro-inflammatory interferon- γ , bacteria or LPS in monocytes (21). It exerts a fungicidal activity towards *C. albicans*. Reduction in the disulphide bonds of human β -defensin 1 increases its antimicrobial activity (22).

Human β -defensin-2 is only expressed in epithelial tissue and is highly upregulated upon stimulation with pro-inflammatory cytokines such as TNF and upon contact with bacterial and fungal pathogens (23) via TLR-2 (24) as well as via IL-1 (25). Psoriatic skin loses its physical integrity thus undermining its role as a mechanical barrier against infection. Nonetheless, it is rarely infected. The strong upregulation of Human β -Defensin-2 in inflamed psoriatic skin (26) can serve as one explanation.

Human β -Defensin-3 is expressed in keratinocytes and airway epithelial cells (27). It is induced via TGF- α – EGFR signalling (25) and exerts its fungicidal activity against *C. albicans* via the same mechanism as the human- β -defensins 1 and 2 (28).

Krishnakumari et al. analysed the anticandidal activity by determining the minimal fungicidal concentration (MFC) of the human β -defensins 1–3 as well as that of their synthetic C-terminal analogs Phd1-3. Of the three defensins, human β -defensins 3 showed the strongest fungicidal activity against *C. albicans* with a MFC of 2.5 μM and HBD-2 the weakest with a MFC of 8 μM . The inactivity of HBD-1 and HBD-2 in the presence of the metabolic inhibitors sodium azide and carbonyl cyanide *m*-chlorophenylhydrazine showed that the function of these two defensins was energy dependent, quite contrary to HBD-3 whose fungicidal capabilities were not altered. The fungicidal activity of all defensins was reduced in an environment of high NaCl, CaCl₂ and MgCl₂. Confocal microscope imaging of *C. albicans* incubated with Phd1-3

showed membrane damage as well as location of Phd1-3 on the outer membrane. A membrane permeabilization assay with Sytox green showed a significant increase in fluorescence after the addition of defensins to *C. albicans*. These results show that at least one mechanism of action of HBD1-3 is increasing the membrane permeability of *C. albicans* (28).

Cathelicidins

Next to defensins, the cathelicidins constitute the other larger group of antimicrobial skin peptides. They are small cationic peptides and possess an N-terminal signal peptide, a highly conserved cathelin domain and a cationic antimicrobial peptide at the C-terminus (3). The cathelin domain has the double function of an antimicrobial peptide and of a protease inhibitor (29). The C-terminus is cleaved by proteases and shows broad, potent antimicrobial activity (3). Cathelicidins are produced by cutaneous and mucosal epithelial cells as well as neutrophils and mast cells (30). The secretion of LL-37 by human eccrine sweat glands is subject to controversy, as Murakami et al. found LL-37 in human sweat of healthy volunteers but Rieg et al. could not (31,32). Moreover, the gene encoding for precursor of human LL-37, *CAMP*, is expressed in keratinocytes during inflammation (33). Members of the cathelicidin family include human LL-37, murine CRAMP and porcine PR-39. They all show fungistatic and fungicidal activity against *Malassezia furfur*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* (34). The minimal inhibitory concentration of LL-37 against two strains of *T. mentagrophytes* and two strains of *T. rubrum* were identical at 12.5 μM , the MFC were 12.5 μM for the two strains of *T. mentagrophytes* and 25 μM against the two strains of *T. rubrum*. LL-37 showed no relevant activity against a strain of *Arthroderma otae*. CRAMP showed a slightly higher antifungal activity at 10 μM against *M. furfur* *in vitro*. *M. furfur* and *T. rubrum* can also slightly increase cathelicidin mRNA expression in human keratinocytes *in vitro*. Cathelicidins CRAMP and LL-37 show fungicidal and fungistatic activity against *C. albicans*, with a similar MIC between 15 and 20 μM . At the skin surface, LL-37 is processed by a serine-protease into shorter peptides such as KS-30 and RK-31. They showed a higher fungicidal activity against *C. albicans* than LL-37 or CRAMP. A Sytox green permeabilization assay showed that LL-37 and RK-31 render the candidal membrane more permeable. Immunohistochemical analysis demonstrated a significant mCRAMP expression after *C. albicans* skin infection *in vivo* (Fig. 1). Furthermore, although mCRAMP-deficient mice showed no increased susceptibility to *C. albicans* in a blood-killing assay, human sweat as well as LL-37 and its products RK-31 and KS-30 showed anticandidal activity, suggesting that cathelicidins are more effective on the skin surface (35).

Synthetic peptides

Kamysz et al. published the antifungal activity of three chemically engineered antifungal peptides against different *Candida spp.* The peptides aurein 1.2, citropin 1.1 A and uperin 3.6 all had lower MICs than amphotericin B and nystatin *in vitro* (36).

Recently, the antifungal activity of two synthetic antifungal cationic peptides, VS2 and VS3 was shown. They irreversibly inhibited the growth of various *Candida spp.*, multidrug resistance strains, *Cryptococcus neoformans*, *Aspergillus niger*, *Fusarium oxysporium* and *Neurospora crassa* at MIC₈₀ values ranging from 15.62 to 250 μM . In combination with fluconazole, non-candidal concentrations of both peptides showed high antifungal activity

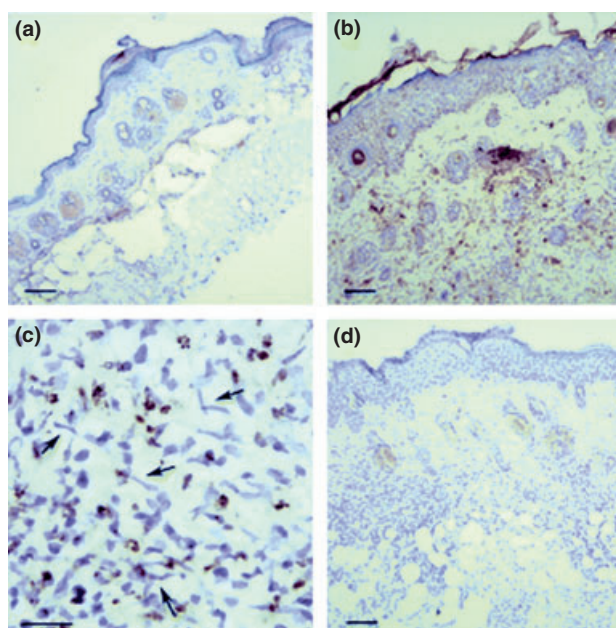


Figure 1. Immunohistochemical analysis of mCRAMP expression in *Candida albicans*-infected mouse skin. (a) Normal uninfected skin and (b–d) *C. albicans*-infected skin. (a–c) Stained with antibody to mCRAMP. (d) Stained with rabbit preimmune serum used as a control. Brown staining in (b) shows increased expression of mCRAMP in *C. albicans*-infected skin compared with uninfected skin in (a). (c) High power (scale bar = 10 mm) of dermis from (b) shows evidence of multiple *C. albicans* organisms indicated by arrows and mCRAMP containing neutrophils [haematoxylin counterstain; scale bar = 40 mm in (a, b, d)] From Fig. 4, (34) (with permission from the nature publishing group).

against a strain of fluconazole-resistant *Candida spp.* In addition to causing a direct permeabilization of the candidal cell membrane, they also induced necrosis by causing an intracellular accumulation of reactive oxygen species and showed fast killing kinetics (37).

A further synthetic peptide, KSL-W attenuated the virulence of *C. albicans* *in vitro*. Pretreatment of *C. albicans* with KSL-W reduced CFUs as well as adhesion on engineered human oral mucosa. The reduction in virulence might also explain the reduction in TLR2, TLR4 and TLR9 expression in engineered human oral mucosa tissue cultivated with KSL-W pretreated *C. albicans*. In contrast, TLR6 expression was increased. KSL-W also downregulated the gene expression of human β -defensins and modulated proinflammatory cytokine secretion. Indeed, KSL-W dose-dependently increased IL-1 β and decreased IL-6 expression of gingival epithelial cells after culture with *C. albicans* *in vitro* (38).

Moreover, recently designed Sap inhibitors based on the known Sap2-substrate specificity data and X-ray analyses of Sap2/inhibitor complexes, taking the structure of the non-selective aspartic proteinase inhibitor pepstatin A as a blueprint. These designed antifungal agents showed high inhibitory activity for Sap1, Sap3, Sap5 and Sap6 (Cadicamo unpublished data).

Dermcidin

Dermcidin is constitutively and specifically expressed in human eccrine sweat glands and is transported to the skin surface. The peptide is proteolyzed after secretion and its processed form, DCD-1L, is highly fungicidal against *C. albicans* under *in vitro* conditions similar to those of human sweat (39). In contrast to many cationic antimicrobial peptides, DCD-derived peptides do not permeabilize microbial membranes, suggesting a different mechanism of action (40).

hGAPDH(2–32)

Recently, a GAPDH-derived peptide from human placental tissue demonstrated antimicrobial activity *in vitro* (Fig. 2). hGAPDH (2–32) is 100% identical to the N-terminus of human GAPDH. It was capable of inhibiting the growth of *C. albicans* in micromolar concentrations *in vitro*, exerting fungicidal activity confirmed by a flow cytometric killing assay. Moreover, it was capable of inhibiting two secreted aspartic proteinases that are important candidal virulence factors and of inducing epithelial IL-8 and GM-CSF secretion in addition to stimulating TLR-4 expression (41).

Targeting virulence factors

Significant improvements in other medical fields such as oncology and transplantational medicine have in turn increased the prevalence of immunocompromised patients. Moreover, increased longevity and a higher hospitalization rate in old age as well as the HIV pandemic have also led to an increase in individuals developing fungal infections. This has in turn led to an increment in antifungal treatments, giving rise to more and more frequent resistances to the drugs employed. We therefore now face an increased necessity to find and develop new antifungal therapies.

One approach has been the exploration of novel targets in fungal pathogens. Antifungal peptides, either synthetic or naturally

occurring have the advantage of having different microbial targets in comparison with classic antifungal agents. Results are promising (37,38). For example, azole resistance in *Candida spp.* can be owing to mutations in genes encoding efflux pumps (42). Indeed, ATP-binding cassette transporter gene mutations such as an over expression of *CDR1* were found to correlate with increasing fluconazole MICs in a series of clinical isolates (43). Many antifungal peptides are small cationic molecules whose target is the fungal outer membrane. Not surprisingly, these peptides can overcome resistances to antifungal drugs currently in first-line use such as fluconazole (37). Moreover, these peptides occur naturally or are similar to natural products, so they can be designed with few side effects on host cells (38). Nonetheless, the most promising application of these antifungal agents seems to be topical and not systemic use (35). The problem of increased drug-resistant, life-threatening systemic fungal infections remains. One possible use of antifungal peptides could therefore be the prophylaxis of systemic fungal infections in populations at risk by strengthening the epithelial barrier as a first line of defence by preventing invasion.

Another strategy is to target fungal virulence factors themselves. One promising target is the biofilm formation, especially of *Candida spp.*, Recently, Mandal et al. (44) published an effective concentration dependant inhibition of *Candida tropicalis* growth and biofilm disruption with the novel antifungal plant peptide Tn-AFP1 extracted from *Trapa natans* fruits.

A further group of fungal pathogenicity factors of interest as therapeutic targets is the family of secreted aspartic proteinases (Saps) of *C. albicans* (45) (Fig. 3). It has been shown that the adhesion capabilities of *C. albicans* can be reduced either by mutations in SAP-coding genes or by using the SAP inhibitor Pepstatin A (47).

Antiretrovirals of the proteinase inhibitor group such as ritonavir can themselves reduce *C. albicans* adhesion (48), a finding corroborated clinically by the finding that patients with HIV under therapy with proteinase inhibitors had a lower oral *Candida* load (49). At first, the reduction in fungal infections, especially of oral candidiasis in patients with HIV treated with antiretrovirals was attributed to an improvement of the immune status. Since 1998, it has been known that antiretroviral proteinase inhibitors

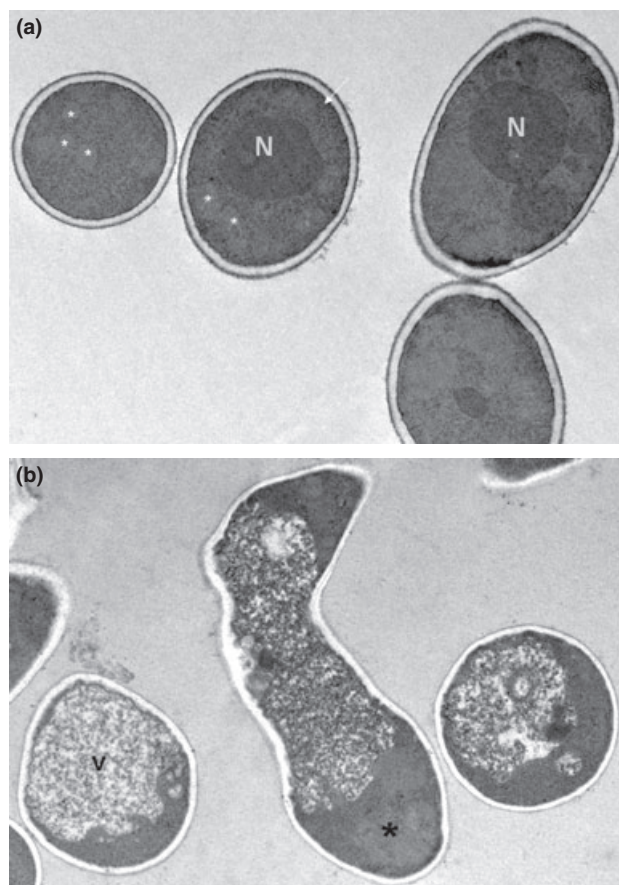


Figure 2. Electron microscopy of *Candida albicans* SC5314 cells grown in 2% Sabouraud glucose medium without (a) and with (b) 125 mg/l hGAPDH (2–32) for 24 h. (a) Cells grown without hGAPDH (2–32) have a regular morphology. N: nuclei, arrow: endocytoplasmic reticulum, *: mitochondria. (b) *C. albicans* grown under the influence of hGAPDH (2–32). v: enlargement of the fungal cytoplasmic vacuoles. *: disorganization of the internal organelles.

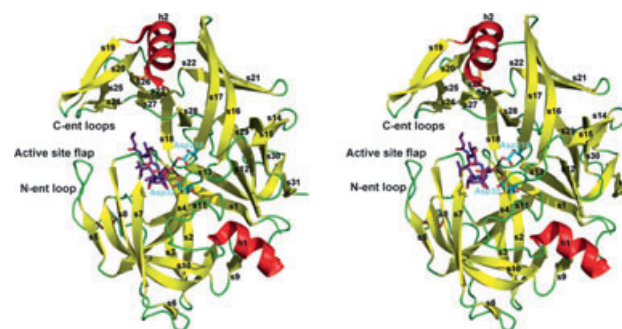


Figure 3. Structure of the Sap5 complexed with pepstatin A. Stereo overall view of Sap5 as example for the overall structure of the Sap isoenzymes. Sap isoenzymes mainly consist of β -strands arranging to a tightly packed, kidney-shaped globular protein. The side chains of the two catalytical aspartates are displayed in blue, the two disulphide bridges (Cys47–Cys59 and Cys256–Cys294) in orange. The loops tied together by the disulphide bridges are specified as N-terminal entrance loop (cysteine residues 47 and 59) (N-ent loop) and C-terminal entrance loops (cysteine residues 256 and 294) (C-ent loops). β -Sheets 7,8 (residues 81–91) represents the active site flap that plays an important role in inhibitor binding. Pepstatin A is shown as a stick model coloured in magenta (46).

Table 1. The following table lists the species, against which an antifungal effect of the specified antimicrobial peptide has been confirmed

Antifungal peptide	Susceptible species	References
RNase-7	<i>Candida albicans</i>	(4)
Lysozyme	<i>C. albicans</i> , <i>Candida parapsilosis</i> , <i>Aspergillus fumigatus</i> , <i>Penicillium spp</i>	(5)
Antileukoprotease	<i>A. fumigatus</i> , <i>C. albicans</i>	(16)
Calprotectin	<i>C. albicans</i>	(17)
Histatins	<i>C. albicans</i>	(19)
Defensins		
Human β -defensin-1	<i>C. albicans</i>	(20,21,28)
Human β -defensin-2	<i>C. albicans</i>	(23,28)
Human β -Defensin-3	<i>C. albicans</i>	(27,28)
Cathelicidins	<i>C. albicans</i> , <i>Malassezia furfur</i> , <i>Trichophyton mentagrophytes</i> , <i>T. rubrum</i>	(31)
Dermcidin	<i>C. albicans</i>	(39)
hGAPDH (2-32)	<i>C. albicans</i>	(41)
Synthetic peptides		
aurein 1.2, citropin	<i>C. albicans</i>	(36)
1.1 A, uperin 3.6		
VS2 and VS3	<i>Candida spp.</i> incl. drug-resistant strains, <i>Cryptococcus neoformans</i> , <i>A. niger</i> , <i>Fusarium oxysporium</i> , <i>Neurospora crassa</i>	(37)
KSL-W	<i>C. albicans</i>	(38)
Lactoferrin	<i>C. albicans</i> , <i>C. glabrata</i>	(55)

such as indinavir and saquinavir can dose dependently inhibit secreted aspartic proteinases (Saps) (50). This was confirmed by Schaller et al. who investigated the inhibitory effect of saquinavir and amprenavir on Saps of three *C. albicans* strains from HIV-infected patients. The results showed significant reduction in their proteolytic activity, comparable with that of the classic Sap inhibi-

tor pepstatin A (51). Borg-von-Zepelin et al. published an article in 1999 demonstrating the ability of four antiretrovirals (ritonavir, saquinavir, nelfinavir and indinavir) to inhibit Sap 1-3 in a dose-dependant manner. Nonetheless, only a slight inhibition of Sap 4–6 with the highest concentration of saquinavir could be achieved. Nelfinavir marginally reduced Sap-6-activity (52). All in all, the further development of protein inhibitors primarily targeting the virulence factors and not the pathogen itself as antifungal therapies seems to be a promising strategy (53).

Another promising development in the same direction has been the design and application of antibodies targeting fungal virulence factors. A targeted therapy against SAP-2 decreased candidal epithelial adhesion on the vaginal wall in a rat model and increased the clearance of the infection (54).

In summary, a better understanding of antifungal peptides will lead to the discovery of novel microbial targets thus aiding in the design of new strategies to keep staying a step ahead in the fight against increasingly drug-resistant fungal infections.

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Conflict of interests

The authors have declared no conflicting interests.

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