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Novel antifungal agents, targets or therapeutic strategies for the treatment of invasive fungal diseases: a review of the literature (2005-2009)

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ABSTRACT

Background: The incidence and prevalence of serious mycoses continues to be a public health problem. Despite aggressive treatment with new or more established licensed antifungal agents, these infections are an important cause of morbidity and mortality, especially in immunocompromised patients.
Aims: To critically review the literature regarding important new developments in the field of antifungal therapy both in the English and Spanish versions.
Methods: The search of the literature focused on different antifungal targets or mechanisms of action as well as new agents or strategies that could improve antifungal therapy.
Results: The review produced a huge amount of information on the use of virulent factors such as growth, filamentation, pathogen tissue clearance, among others, as putative targets of antifungal activity. More recently, the chemical-genetic relationships for licensed agents as well as for other compounds have been provided by the identification of the genes related to the mechanism of action.
Conclusions: Although the antifungal activity of numerous compounds has been examined, most of them are at the *in vitro* or animal models of efficacy stages. Therefore, further investigation should be carried out to realize the true clinical utility of these compounds.

Nuevos antifúngicos, nuevas dianas y estrategias terapéuticas para el tratamiento de las micosis invasoras: revisión de la bibliografía (2005-2009)

Palabras clave:
Concentraciones antifúngicas
Equinocandinas
Estudios genéticos
Nuevas dianas antifúngicas
Nuevas estrategias terapéuticas antifúngicas
Nuevos antifúngicos

RESUMEN

Antecedentes: La incidencia y la prevalencia de micosis invasoras continúa siendo un problema de salud pública. A pesar de los tratamientos más agresivos con los nuevos fármacos o los antifúngicos más establecidos, las infecciones fúngicas causan bastante mortalidad y morbilidad, especialmente en los pacientes inmunodeficientes.
Objetivos: Revisar críticamente la bibliografía acerca de los nuevos desarrollos más importantes en el campo del tratamiento antifúngico en las versiones en español y en inglés.
Métodos: Se enfocó la revisión en los estudios relacionados a dianas o mecanismos de acción diferentes a los actuales; también se revisaron los informes de fármacos nuevos, estrategias terapéuticas prometedoras o alternativas para los pacientes que presentan infecciones fúngicas invasoras.
Resultados: En numerosos estudios se ha evaluado una variedad de factores de virulencia como posibles dianas de actividad antifúngica. Más recientemente, la relación química-genética de los antifúngicos aprobados y de otras moléculas se ha definido debido a la identificación de los genes relacionados con el mecanismo de acción correspondiente.
Conclusiones: A pesar de los resultados favorables aportados en esos estudios, el desarrollo de la mayoría de estas moléculas está al nivel de su espectro in vitro o in vivo, pero en estudios de eficacia en modelos animales. Por lo tanto, deben realizarse más evaluaciones para que su desarrollo llegue al nivel de ensayos clínicos.

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The incidence and prevalence of invasive fungal infections have increased since the 1980s, especially in the large population of immunocompromised patients and/or those hospitalized with serious underlying diseases[7,24]. In addition, the mortality and morbidity of these infections is quite substantial. The most common fungal pathogens continue to be the species of *Candida* and *Aspergillus*[7,54,86,91]. Parallel to the increase in fungal infections, two triazoles (voriconazole and posaconazole) and three echinocandins (anidulafungin, caspofungin and micafungin) have been licensed for the treatment and prevention of these infections[4-6].

The echinocandins have a unique mechanism of action (inhibition of β-1,3-D-glucan synthase) and a broad and similar spectrum of *in vitro* activity against *Candida* spp. and *Aspergillus* spp.[25,66,85]. During the last few years, mechanisms of resistance to most licensed agents in *Candida* spp., and to a certain point in *Aspergillus* spp., have been elucidated[25,46,83]. Although resistance of common *Candida* spp. and *Aspergillus* spp. to echinocandins and azoles is rare, it has been documented and continues to be reported[8,25,29,46,83]. The mortality rates associated with invasive candidiasis are approximately 0.4 deaths per 100,000 population/year while there was a decrease with aspergillosis from 0.42 per 100,000 in 1997 to 0.25 per 100,000 in 2003 in the United States[86]. Although it is hoped that the introduction of these new agents will improve these rates, the mortality rate in most aspergillosis studies is about 50%. Therefore, there is a need for new targets or strategies in antifungal therapy. This review summarizes some of the new developments and/or discoveries found in the literature since 2005 (Table 1).

Antifungal agents under development

New polyene and other agents

The lipopeptide micafungin (8, FK463)[103], like the other echinocandins, has fewer side effects than amphotericin B and other agents, but the echinocandins have not been approved as the first line therapy for invasive aspergillosis. The novel polyene SPK-843 showed less renal toxicity than both amphotericin B or liposomal amphotericin B and also better activity than micafungin and both established polyenes in a murine model of pulmonary aspergillosis[45]. Clinical trials are presently being conducted. Preclinical *in vitro* and *in vivo* evaluations of the novel arylamidine T-2307 indicate that this agent has potential for the treatment of candidiasis, cryptococcosis and aspergillosis. The mechanism of action of T-2307 is not yet established, but it has been suggested that it is associated with the mitochondrial function of the fungal cell[68]. These preliminary results support the continued development of these compounds.

An ambruticin analog (a cyclopropyl-pyran acid which interferes with the osmoregulatory system) was effective in both murine models of coccidioidomycosis[94] and pulmonary aspergillosis[19], but no further information was found in the literature since 2006.

New triazole

Voriconazole has no activity against the mucoraceous. The new triazole isavuconazole, (BAL4815) in late state clinical development for the treatment of aspergillosis, appears to have *in vitro* activity against the zygomycetes (MIC_{50} and MIC_{100} of 1 and 2 μg/ml, respectively) versus voriconazole MICs of ≥ 16 μg/ml[32]; also, its activity was superior to that of both itraconazole and voriconazole against *Candida* spp.[92]. However, contradictory results have been documented for the zygomycetes in other studies ($MICs_{50}$ of > 6 μg/ml)[60,82].

Icofungipen

Icofungipen (PLD-118, BAY 10-8888) is a derivative of cispentacin. It is a beta amino acid that targets isoleucyl-t-RNA synthetase; intracellular inhibitory concentrations at the target site are achieved by its active accumulation in susceptible fungal cells. Although its *in vitro* activity against *Candida albicans* is poor, it has shown strong *in vivo* activity in a neutropenic rabbit model for disseminated candidiasis, including the treatment of central nervous system infection[36,84]. It has dose-dependent pharmacokinetics and it shows potential for the treatment of invasive candidiasis.

Inhibitor of β-1,6-glucan synthesis

75-4590, a pyridobenzimidazole, is a specific inhibitor of β-1,6-glucan synthase; it has shown activity against *Candida* spp. and appears to inhibit hyphal elongation of *C. albicans*[50]. Genetic analysis of a resistant mutant of *Saccharomyces cerevisiae* indicated that its primary target was Kre6p (a β-1,6-glucan synthase)[70]. Its growth inhibition is dose-dependent; since Kre6p homologous have been found in *Aspergillus fumigatus*, partial silencing of *KRE6P* expression makes *A. fumigatus* more susceptible to Congo red which appears to indicate the role of Kre6p in cell wall construction[37].

Monoclonal antibody therapy

Patient therapy

Casadevall[13] considers serum therapy the third age of antimicrobial therapy. In 2006, Pachl et al[77] reported the results of the combination of amphotericin B and Mycograb (*Neu*tec Pharma), a human recombinant monoclonal antibody as an inhibitor of heat shock protein 90, in patients with invasive candidiasis. An 84% overall response was observed by day 10 in the combined therapy versus 48% in patients treated with amphotericin B alone; clinical and mycological response, *Candida*-attributable mortality and rate of culture-confirmed sterilization were also superior with the combined therapy. The first application of monoclonal antibody therapy for a fungal disease in humans was the evaluation of the murine-derived anticryptococcal antibody 18B7 for cryptococcal meningitis by Larsen et al[53]. Their promising results support further evaluation of 18B7.

Animal models in vitro

The monoclonal antibody Mab C7 has been shown to inhibit the adhesion and germination of *C. albicans* and has direct candidacidal activity[75]. The use of microbe-specific monoclonal antibodies as delivery vehicles for targeting biofilms with cytocidal radiation was successfully evaluated by Martinez et al[61]; they found that *Cryptococcus neoformans* biofilms were susceptible to this treatment, which could be a novel option for either the prevention or treatment of biofilms. More recently, the combination of caspofungin and efungumab, a human antibody fragment, was used against the heat shock protein 90, a target of the human response in invasive candidiasis[39]; these preliminary results indicate that efungumab enhanced the activity of caspofungin in the animal model. Similar results were obtained by Mattila et al[63] in an immunosuppressed murine model of invasive pulmonary *A. fumigatus* infection when animals were treated with Dectin-1 Fc via beta-glucan recognition and opsonic elimination; the conclusion was that Dectin-1 Fc could serve as a prophylactic treatment of this infection.

Strategies for the treatment of biofilms

C. albicans biofilms are intrinsically resistant to most antifungal agents. The optimal efficacies of caspofungin and micafungin were evaluated using an *in vitro* model of *C. albicans* biofilm[14]. Caspofungin (2 mg/ml) and micafungin (5 mg/ml) could be good candidates for the reduction or control of fungal biofilms associated with silicone medical devices, as part of the antifungal lock. Both echinocandins

Table 1
New antifungal agents, targets, strategies (2005-2009)

Agent, strategy	Mode of action, antifungal target, result	Stage of development	Reference
New antifungal agents			
Polyene SPK-843	Membrane ergosterol	Clinical trials	45
Arylamidine T-2307	Possible mitochondrial	Preclinical	68
Ambruticin analog	Osmoregulatory system	Preclinical	19,94
Isavuconazole	Ergosterol inhibition	Clinical trials	32,60,82,92
Icofungipen	Isoleucyl-t-RNA synthase	Preclinical	36,84
Pyridobenzimidazole 75-4590	β-1,6-glucan synthase	In vitro	37,50,70
Monoclonal therapy			
Monoclonal (Mycograb and anticryptococcal antibody 18B7)	Enhanced antifungal therapy	Clinical trials	53, 77
Mab C7	Adhesion, germination inhibition	Animal model	75
Monoclonal antibody as cytocidal radiation vehicle	Biofilm damage	In vitro	61
Efungumab (antibody fragment)	Enhanced antifungal therapy	Animal model	39
Dectin-1Fc	Innate defense augmentation	Animal model	63
***Candida* biofilm**			
Micafungin+caspofungin	Synergy	In vitro	14
Terpenes	Potential biofilm treatment	In vitro	21
Baicalein	Possible mRNA lowering	In vitro	12
Synergism			
Micafungin+ amphotericin B or + flucytosine	Synergy	In vitro, animal models	23,79,93
Voriconazole+lovastatin	Synergy	In vitro, animal models	16
Azoles+retigeric acid	Synergy	In vitro	98
Caspofungin+calcineurin	Synergy-cell wall inhibitors	In vitro	97
Amphotericin+EDTA	Synergy	Animal model	35
Micafungin+flucytosine-pharmacokinetic profile	Maximal tolerated dose	Clinical	38
Micafungin+voriconazole-pharmacokinetics	No interference, no synergy	Healthy adults	49
Drug monitoring, pharmacodynamic and pharmacokinetic studies			
Voriconazole, itraconazole and posaconazole drug monitoring	Inter-subject variation	Clinical	3,28,80,96
Flucytosine drug monitoring	Maximal killing	Animal models	3,41
Flucytosine, voriconazole-drug monitoring	Toxicity	Clinical	3,80
Micafungin pharmacodynamic target	Efficacy dosing regimen: same for *C. albicans*, *C. glabrata*	Animal model	2
Micafungin dose adjustment	Weekly versus daily: same	Animal model	33
Micafungin dose level	Initial oral dose efficacy	Clinical	76
Micafungin population-pharmacokinetics	Inter-patient variability-pediatric and adult patients	Clinical	34,40
Peptides versus yeasts			
Galanin message-associated, amentoflavone, lactoferrin	Hyphal-pseudohyphae formation inhibition	Preclinical	44,58,87
Histidine H2K4b	Growth inhibition	Preclinical	114
Histatin 3 and 5	Targets: elongation factor 2, cell wall binding as targets	Preclinical	26,42
Cathelicidin, porcine, and bass peptides	Membrane permeabilization	Preclinical	9,10,100
Fimbrigal-P, surfactant cationic-nanoparticles and lipids	Adhesion inhibition	Preclinical	27,64,105
Apigenin, kaempferol, ibogaine, berberine	Penetration/adhesion inhibition	Preclinical	111
Human beta-defensins (HBD-1 to HBD-3) and analogs	Mechanism not understood	Preclinical	51
Defensins: plant (RsAFP2) and others	Not determined; potential prophylactics and others	Preclinical	1,55,67,101
Oligopeptides and aminoacids	Cdr1p, Cdr2p overexpression (higher uptake rate)	Preclinical	106
Peptides versus moulds			
Lipopeptide palmitoyl-lys-$_D$ala-Lys	Potential detergent-like effect	Preclinical	104
Heat-stable antifungal factor (HSAF)	Sphingolipid synthesis disruption	Preclinical	112
Antifungal protein PAF	Membrane hyperpolarization, ion channel activation	Preclinical	62
Hexapeptide PAF26	Polar growth and branching alteration	Preclinical	69
Xanthorrhizol, lectin	Conidial germination inhibition	Preclinical	73,89
Drosomycin-like defensin (DLD)	Immunoregulatory effect; DLD mRNA expression	Preclinical	95
Enzyme inhibitors			
Synthase and other inhibitors	Enzyme inhibition	Preclinical	43,65,74,81,88,109
Chitin synthase inhibitors	Chitinase inhibition, chitin hydrolysis	Preclinical	18,47,48,110
Bisquaternary salts	Phospholipase inhibition	Preclinical	72
Miltefosine	Potential phospholipase inhibition	Preclinical	107
Other targets			
Coumarin	Cytochrome biosynthesis disruption	Preclinical	102
Indol-3-carbinol	DNA binding	Preclinical	99
Cruentaren	Mitochondrial ATPase inhibition	Preclinical	52
Fatty acids			
Whey derived free fatty acids, lavender oil, others	Beta-oxidation pathway blocking; germination, hyphal elongation inhibition	Preclinical	11,20,22,56
Genetic studies			
Gene screening	Hyphal growth inhibition	Preclinical	108
Gene modulation and doxycycline	Filamentation inhibition	Preclinical	90
Gene transfer and chitotriosidase	Chitin inhibition	Preclinical	31
MET2 gene (encodes homoserine transacetylase, HTA)	*C. neoformans* HTA target	Preclinical	71
MET3 promoter system	*C. albicans* essential genes	Preclinical	57
Gene suppression (*FAS1, FSA2*)	Fatty acid synthase inhibition	Preclinical	17

were able to significantly and persistently reduce the yeast metabolic activity of intermediate and mature biofilms, 12 h and 5 days old, respectively, when used as catheter lock solutions. The *in vitro* activity of terpenes[21] and baicalein[12] has also been evaluated against *C. albicans* biofilms and they appear to be promising candidates to either treat or reduce the incidence of device-associated infections. The cells treated with baicalein expressed lower levels of mRNA than the cells grown in its absence[12].

Synergism

Antifungal drug-drug combinations

The echinocandins do not have any activity against *C. neoformans*. The *in vitro* interactions of micafungin with either amphotericin B, fluconazole, itraconazole or voriconazole were evaluated for different *Cryptococcus* spp.; no antagonism was observed and synergy was frequently observed with the combination of micafungin and amphotericin B[93]; similar results were observed in experimental aspergillosis[23] with the same combination and more recently, against simulated *Candida* endocarditis vegetations with the combination of micafungin and flucytosine[79]. More research is needed regarding the combinations of echinocandins with triazoles and lipid formulations in randomized clinical trials. Although the combination of caspofungin with these latter agents has provided mostly favorable results, they were not obtained in randomized clinical trials[113].

Antifungal drug combination with other agents

The combination of the statin lovastatin and voriconazole was synergistic both *in vitro* and *in vivo* in a fly *Drosophila melanogaster* model of zygomycosis[16]. More recently, favorable *in vitro* data has been reported for retigeric acid either alone or in combination with azoles against *C. albicans*[98]. Steinbach et al[97] demonstrated, using a calcineurin A mutant (*cnaA*), that calcineurin is critical for *A. fumigatus* hyphal growth, tissue invasion and pathogenicity and enhanced the antifungal activity of cell wall inhibitors such as caspofungin or nikkomycin. EDTA, a lead poisoning chelator therapeutic that appears to have antifungal activity, was shown to have synergistic activity in combination with amphotericin B lipid complex in a rat model of immunosuppressed *A. fumigatus* invasive pulmonary aspergillosis[35]. The clinical significance of these observations is yet to be determined.

Pharmacokinetic studies

A pharmacokinetic study was conducted to determine the maximal tolerated dose of micafungin, and especially the pharmacokinetic profile when micafungin was combined with fluconazole in cancer patients undergoing either bone marrow or peripheral stem cell transplants[38]. This combination was found to be safe and the maximal tolerated dose of micafungin was not reached at 200 mg/day for four weeks. Keirns et al[49] reported that voriconazole did not affect the pharmacokinetics of micafungin; however, an absence of drug interaction was observed in healthy adults. These are promising results, but data from patients are needed.

Drug monitoring, pharmacodynamics and pharmacokinetic strategies

Drug monitoring

Therapeutic monitoring is essential to ensure drug exposure (dosage increase when it is possible) or to avoid toxicity (administer lower doses) during the antifungal treatment of invasive mycoses. Monitoring of voriconazole serum concentrations is important due to the frequent inter-subject variability (trough concentrations of <0.1 to about 10 μg/ml from patients taking 200 mg twice a day)[96]. There was a 90% response to voriconazole therapy when serum levels were >1 μg/ml, but only 54% when the serum concentrations were lower in patients with invasive candidiasis or aspergillosis[80]. Based on those results, the paucity of voriconazole MIC data for *Histoplasma capsulatum*, the lack of prospective trials to establish the effectiveness of this agent for histoplasmosis treatment and the wide range of voriconazole serum concentrations (<2.05 to<0.125 μg/ml) also found in their study, Freifeld et al[28] have recommended measuring trough levels in patients receiving voriconazole for histoplasmosis. As there is also inter-subjective variability of itraconazole and posaconazole serum concentrations, drug monitoring of these triazoles also could be useful[3]. Maximal organism killing has correlated with flucytosine concentrations above the MIC in animal models[3,41]. In addition, high flucytosine levels have correlated with toxicity and elevated voriconazole concentrations with encephalopathy[3,80].

Pharmacodynamics and pharmacokinetics

Pharmacodynamic results indicated that the current clinical dosing regimens of micafungin were appropriate for the treatment of infections caused by both *C. albicans* and *Candida glabrata*; micafungin exposures needed for efficacy were similar[2]. Relating the results in the murine neutropenic candidiasis model to human micafungin pharmacokinetics for the 100 mg/day dosing regimen would predict an inhibitory pharmacodynamic target against both species with MICs up to 0.06 μg/ml. In addition, the free drug micafungin exposures required to produce stasis and killing endpoints were similar to those reported for anidulafungin against *C. albicans* and *C. glabrata*[2]. Other strategies regarding dosing regimen adjustment to improve micafungin efficacy also have been examined in a murine neutropenic model of candidiasis[33] and in patients[76]. Furthermore, population studies have provided real inter-patient (pediatric and adult) pharmacokinetic variability[34,40].

Serum effect on antifungal activity

Serum-MICs of both caspofungin and micafungin for *C. albicans* were better predictors of *in vivo* potency than conventional MICs (hyphal growth inhibition or *C. albicans* kidney burden measurement)[59]. These results were confirmed recently by the reports of the influence of serum in drug protein binding. Using *in vitro* growth assays, it has been reported that protein binding shifted the antifungal activity of echinocandins against *Aspergillus* spp. and *Candida* spp. resulting in nearly equivalent MICs or MECs[78]; serum decreased the sensitivity of glucan synthase to echinocandins. Because of that, it has been suggested that the susceptible breakpoint established by the Clinical and Laboratory Standards Institute of $\geq$ 2 μg/ml does not apply to the three echinocandins, but only to caspofungin. Using *fks1* mutants, Garcia-Effron et al[30] have demonstrated that serum MICs captured all (100%) *fks1* mutants above the MIC breakpoint, but this breakpoint was less applicable for anidulafungin and micafungin. Micafungin or anidulafungin MICs it should be equal or greater than 0.5 μg/ml provided similar results (95% of the mutant isolates were captured). Their recommendation was to either lower the breakpoint or to use caspofungin in vitro data as a surrogate marker to identify echinocandin resistance, since the three echinocandins have similar activity target, resistance mechanisms, spectrum and *in vitro* potency; the use of surrogates has been previously suggested for the triazoles, where fluconazole breakpoints can be used to assess patterns of susceptibility of other triazoles.

Peptides

Further of research has been dedicated to the investigation of the antifungal activity of a variety of peptides mostly against *C. albicans*,

A. fumigatus and *C. neoformans*. Although they are promising leads for the development of new agents, a great deal of investigation is needed to determine their clinical usefulness. Some of the developments in this area are summarized below.

Activity against C. albicans *and* C. neoformans

Inhibition of the transformation from budding to hyphal or pseudohyphae formation, an important virulent factor in *C. albicans*, has been observed with the galanin message-associated peptide (GMAP)[87], amentoflavone[44] and a lactoferrin-derived peptide[58]; lactoferrin activity was dose-dependent and it was effective in disseminated murine candidiasis

The histatins have potential as antifungal agents since they are the first line of defense against infection with oral candidiasis. Zhu et al[114] synthesized a four-branched histidine (H2K4b) that affected the growth of several species of *Candida* by pH buffering followed by endosomal-disruption. Since this molecule accumulated efficiently in *C. albicans*, it may indicate its ability to transport other antifungal agents. Histatin resistant derivatives of *C. albicans* had the same killing mechanism as the parent strain, but they had different proteins than those found in the parent cell; the most important of those differences was the absence in the resistant derivatives of the elongation factor 2 (Ef2), a specific target for the antifungal sordarin. There was also a decrease in the transcript level of the potassium transporter encoded by *TRK1*, a critical mediator of histatin killing. These results indicate that there may be several intracellular targets for histatin 3 in *C. albicans*[26]. Among at least 50 histatin peptides derived from posttranslational proteolytic processing, histatin 5 (Hst 5) has shown the highest level of activity against *C. albicans*. Its mechanism of action involved, first binding to the cell wall protein Ssa2 of *C. albicans,* followed by translocation to intracellular targets. Jang et al[42] demonstrated that binding and transportation were independent events and that the P-113 fragment of Hst 5 required a specific peptide sequence for translocation.

Cathelicidin peptides were shown to have killing activity against *C. albicans* and *C. neoformans* that was associated with membrane permeabilization, but they had little activity against moulds[9]. However, the porcine 1905-Da cationic proline-rich peptide (SP-B) has shown activity against both yeast species and also *A. fumigatus*[10]. Recently, it was demonstrated that the fungicidal activity of the bass peptide derivative piscidin 2 (P2) was based on the formation of pores in the fungal membrane[100].

Several investigators have focused their research on the adhesion and penetration of *C. albicans* in tissues. Foldvari et al[27] demonstrated in a rat model of oral candidiasis that Fimbrigal-P (an antiadhesion synthetic carbohydrate) reduced fungal burden and was a promising antifungal agent for the prevention and treatment of infections when the target was β-GalNac(1-4)-β-alactosidase disaccharide. The surfactant-coated cationic nanoparticles and lipids are potential prophylactics that act by priming the buccal epithelial cells against fungal adhesion and infection[64,105]. The activity of two flavonoid compounds (apigenin and kaempferol), the indole alkaloids ibogaine and berberine were evaluated as potential inhibitors of the virulent factors responsible for the penetration of *C. albicans* into human cells; they appeared to inhibit adherence and had aspartyl proteinase activity. The application of these compounds in cutaneous infection was shown to suppress symptoms and accelerated the elimination of the pathogen from the infection site[111].

The human beta-defensins HBD-1 to HBD-3 and their analogs phd1 to phd-3 have shown fungicidal activity against *C. albicans*. Although the mechanism of action is not yet understood (the initial site of action is the fungal membrane), both analogs may be potential antifungal therapeutic agents[51]. A plant defensin (RsAFP2), not toxic to mammalian cells, has been found to be prophylactically effective against murine candidiasis[101]. Active research continues regarding the characterization of other defensins as possible antifungal agents[1,55,67].

It was demonstrated that the susceptibility to oligopeptides and amino acids was enhanced in *C. albicans* over expressing Cdr1p and Cdr2p, which resulted in higher uptake rates of these peptides via oligopeptide permeases[106].

Activity against A. fumigatus *and other moulds*

Vallon-Eberhard et al[104] have described that the ultra-short lipopeptide, palmitoyl-lys-ala-$_D$ala-Lys (linked to fatty acids) was superior to amphotericin B in an immunosuppressed murine model of invasive pulmonary aspergillosis by *A. fumigatus*, which highlighted the potential of this family of lipopeptides as antifungal agents. Although enough data are not available regarding its mechanisms of action, it was suggested that the activity is membranolytic (detergent-like effect), similar to that of other enzyme inhibitors, e.g., echinocandins. Yu et al[112] reported the antifungal activity of a heat-stable antifungal factor (HSAF) against a variety of fungal pathogens; its target is the disruption of the biosynthesis of sphingolipids, essential but different components of fungal and mammal cells. The *Penicillium chrysogenum* antifungal protein (PAF) elicited hyperpolarization of the plasma membrane and the activation of ion channels[62]. The small hexapeptide PAF26 altered hyphal morphology (polar growth and branching), chitin deposition and caused other detrimental effects[69]; this peptide had preferential activity against moulds. Conidial germination of *Aspergillus* spp. and other moulds was shown to be inhibited by xanthorrhizol[89] and lectin[73].

A drosomycin-like defensin (DLD), a human homologue of drosomycin from the fly *D. melanogaster*, showed specific antifungal activity against filamentous fungi. Both an immunoregulatory effect on *Aspergillus*-stimulated cytokine production and the expression of DLD mRNA in mostly skin human tissues were observed, which is consistent with its putative role as a defensin against invading microorganisms[95].

Enzyme inhibitors

Synthases and other enzymatic targets

Other possible antifungal agents are the synthase inhibitors such as pleofungins (inositol phosphorylceramide)[109], N-alkyl derivatives that inhibit glucosamine-6p synthase[65], elastase inhibitor from *A. flavus* (AFLEI) in combination with other existent licensed agents[74], the GMP synthase inhibitors in *C. albicans* and *A. fumigatus*[88] and the inhibition of mRNA polyadenosine polymerase[43,81] by the natural products parnafungins; these inhibitors deserve further investigation for potential clinical use.

Chitin synthase inhibitors

The cell wall components chitinases are essential for cell wall plasticity during growth. Recently, the *in vitro* antifungal activity of the acidic mammalian chitinase against *C. albicans* and *A. fumigatus* was demonstrated; efficient hydrolysis of chitin was observed[18]. These results confirmed earlier observations regarding the antifungal *in vitro* activity against a variety of fungal pathogens of other natural chitin synthase inhibitors such as sesquiterpene furan compound C-J-01[110], O-methyl pisiferic acid and 8,20-dihydroxy-9(11),13-abietadien-12-one and 2'-benzoyloxycinnamaldehyde[47,48].

Phospholipases

Other possible targets for drug development are the phospholipase inhibitors; inhibition of *C. neoformans* by bisquaternary ammonium salts correlated with the inhibition of cryptococcal phospholi-

pase B1 (PLB1, a newly identified virulent factor); *C. albicans* was also inhibited[72]. On the other hand, Widmer et al[107] found that miltefosine delayed *C. neoformans* infection and mortality and reduced brain burden in a murine model of cryptococcosis; however, the relatively low inhibitory effect on the phospholipase B1 enzyme at concentrations exceeding the MIC by 2 to 20 times suggested that there was another mechanism involved in addition to phospholipase inhibition.

Other targets

Disruption of cytochrome biosynthesis which could induce apoptosis by coumarin derivatives in *C. albicans*[102] and the candidacidal activity of Indol-3-carbinol by binding fungal DNA[99] are two different mechanisms of action. Cruentaren has shown an inhibitory effect on mitochondrial ATPase activity as well as the growth of some yeasts and moulds[52]. Chamilos et al[15] have shown that caspofungin MICs were lower when the *C. parapsilosis* mitochondrial respiratory pathway was inhibited; therefore this pathway could be responsible for the decreased susceptibility of this species to caspofungin and other echinocandins.

Fatty acids

The antifungal activity of fatty acids has been recognized for years. Although some of them are used as topical over-the-counter formulations, several fatty acids were evaluated between 2006 and 2008 for either topical use (6-acetylenic acids)[56] or for the treatment of more invasive mycoses, e.g., (+/-)-2-methoxy-4-thiatetradecanoic and (+/–)-2- hydroxyl-4-thiatetradecanoic acids blocked the beta-oxidation pathway of *C. albicans* and *C. neoformans*[11], and whey-derived free fatty acids[20] and lavender oil[22] inhibited germination or hyphal elongation of *C. albicans*.

Discovery of antifungal targets by genetic studies

The application of chemically induced haplo-insufficiency (growth phenotypes associated with the loss or deletion of function) has been used to screen for genes involved in the hyphal growth of *C. albicans*[108], as well as to investigate fungal viability and virulence of other species; this type of research has led to the discovery of many putative antifungal targets.

Saville et al[90] genetically engineered a *C. albicans tet-NRG1* strain in which they could modulate filamentation and virulence by the presence or absence of doxycycline. They were able to confirm that this species can only cause disease when filamentation was induced with doxycycline. Doxycycline removal led to increased survival; mortality rates also increased markedly the longer the intervention was delayed. It was concluded that filamentation inhibition could be targeted to treat disseminated candidiasis.

Chitotriosidase, which is secreted by human macrophages, has been associated with the defense against chitin-bearing pathogens. The engineered cells (gene transfer of the chitotriosidase gene into Chinese hamster ovary cells) inhibited growth *in vitro* of *Aspergillus niger*, *C. albicans* and *C. neoformans* and increased longevity in a murine model of *C. neoformans*[31]. This effect was possible by the prolonged delivery of recombinant chitotriosidase.

Nazi et al[71] identified the MET2gene (required for virulence) of *C. neoformans* H99 that encoded HTA (homoserine transacetylase) by complementation of an *Escherichia coli metA* mutant that lacks the gene encoding homoserine trans-succinylase (HTS). By screening a 1,000-compound library for HTA inhibitors, the first antifungal inhibitor of HTA was identified; this identification validated the use of fungal HTA as a potential target of new antifungal agents.

Using a genome comparison tool, Liu et al[57] identified 240 conserved genes as possible antifungal targets in ten fungal genomes; essential genes in *C. albicans* were then identified by a repressible MET3 promoter system. When the expression of the *C. albicans ERG-1*target was reduced via down-regulation of the *MET3* promoter, the mutant became hypersensitive to its terbinafine inhibitor. Antifungal target candidates can be screened by this process.

It has been reported that fluconazole potency against *C. neoformans* was enhanced and became fungicidal when the expression of the genes (*FAS1* or *FAS2*) that encoded *C. neoformans* fatty acid synthase was suppressed[17]; these observations indicated that fatty acids were essential for *C. neoformans* in vitro and in vivo growth. Therefore, *FAS1* and *FAS2* can potentially be fungicidal targets for *C. neoformans* either alone or combined with azoles. Again further development is needed.

Conclusions

Although much progress has been accomplished towards the identification and understanding of putative targets or mechanisms of action that could lead to the development of new and improved antifungal agents, the usefulness of these compounds can only be assessed in randomized clinical trials.

Author's disclosure

The author has nothing to declare.

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