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The GPI anchor pathway: a promising antifungal target?

“The activity of Gwt1 inhibitors as well as Mcd4 inhibitors versus a broad range of yeast and molds and lack of observed cross-resistance with existing therapeutics should encourage further investigation for novel antifungal leads targeting GPI biosynthesis.”

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Keywords: antifungal • fungal infection • glycosylphosphatidylinositol • invasive • mannanprotein • mycosis • resistance • synergy

A key challenge with developing antifungal drugs is that both humans and fungi are eukaryotic organisms; as a result, antifungal therapeutics may have substantial toxicity due to inhibition of closely related human targets. Compounding this challenge, the principal clinically relevant fungal pathogens *Aspergillus fumigatus* and *Candida albicans* diverged from a common ancestor 1 billion years ago [1], underscoring their substantial genetic and pathogenic diversity and the difficulty of new antifungal agents achieving broad-spectrum activity. Identifying therapeutics that can both overcome this diversity as well as remain well tolerated in human subjects has proven elusive. As a result, there are only three mechanistically distinct classes of antifungal therapeutics that are currently employed to treat serious life-threatening infections due to both yeast and molds.

There have been multiple ‘calls to action’ for the development of new antifungal therapeutics and many discussions of what the optimal properties of the ‘ideal’ therapeutic might be: 1) activity against fungi that are intrinsically resistant to currently marketed antifungal agents; 2) broad-spectrum activity, especially versus a wide array of molds that are difficult to diagnose in the clinic; 3) no cross resistance with current antifungals; 4) the ability to bolster the immune response to combat mycoses; and 5) fungicidal activity [2,3]. Within this framework, recent work on inhibitors of glycosylphosphatidylinositol (GPI) biosynthesis will be considered for their potential as new entrants in the antifungal armamentarium.

With the increased prevalence of drug-resistant species of both yeasts and molds as causative pathogens of invasive, life-threatening infections, there have been renewed efforts to identify novel, broad-spectrum antifungal agents that can provide therapeutic benefit to improve clinical outcomes. GPI biosynthesis is a conserved and essential process that is required for cell wall biogenesis in both yeasts and molds [4,5]. The role of GPI in anchoring proteins to the outer wall of fungi underscores its function in maintaining cell wall integrity [6]. Due to the prevalence of this pathway in fungal species, GPI biosynthesis presents an attractive target for the development of a broad-spectrum antifungal compound that is also a cell wall active agent. Indeed, numerous cell wall anchored proteins have been identified that rely on the GPI post-translational modification for cell surface localization (*Saccharomyces cerevisiae* has ~60) and chemical genetics approaches have proven highly successful in identifying these GPI-anchored proteins as well as cognate small molecule inhibitors of GPI biosynthesis [7,8]. The structures of known GPI pathway inhibitors described herein as well as a representation of the GPI precursor and enzymatic steps of GPI biosynthesis are shown in Figure 1. However, as orthologous GPI biosynthetic enzymes exist in man, fungal specificity of such agents remains an important consideration.
One antifungal drug target identified by chemical genetics that is part of the GPI pathway is Gwt1, an enzyme that is required for the assembly of GPI-anchored proteins that are later attached to the fungal cell wall [6]. This enzyme has been further characterized as an acyltransferase that is required for the production of acylated GPI. Importantly, acylated GPI forms a required anchor that attaches mannoproteins to the cell wall of yeasts including *C. albicans* [79]. This target has been explored by the pharmaceutical company Eisai Co. Ltd because of: 1) low homology (<30% amino acid sequence identity) with the closest mammalian ortholog, PIG-W; 2) the critical role of Gwt1 in maintaining the integrity of the fungal cell wall; 3) the role of Gwt1 in enabling fungal cell adhesion to host mucosal surfaces; 4) the potential to avoid cross-resistance with existing antifungal therapeutics [10]. A challenge with producing a rationally designed small molecule Gwt1 inhibitor is
due to the lack of 3D structural elucidation of the target, likely because Gwt1 has an estimated 13 transmembrane domains [9]. As a result, the drug discovery effort at Eisai Co. started with a chemical library screen employing a newly developed reporter assay for localization of the S. cerevisiae GPI-anchored mannoprotein, Cwp2, to the cell wall. After screening their corporate library, the hit compound 1-[4-butyl-benzyl-isooquinoline] (BIQ1) was identified [7]. Analog design was then based on (BIQ1) as an inhibitor of Gwt1 [7], and these analogs were refined by phenotypic screening for target engagement and antifungal activity both in vitro and in vivo [10].

This extensive effort yielded a lead compound, E1210 (now known as APX001A), that has shown promising broad-spectrum activity against both yeasts and molds, and was well tolerated when administered to mice at therapeutic doses [10]. Importantly, E1210 exhibited no measurable inhibition of PIG-W, the closest human ortholog of fungal Gwt1. E1210 also exhibited >40% oral bioavailability in multiple mammalian species [12]. Moreover, this compound has shown promising activity versus fungal pathogens that have proven challenging to treat with the existing antifungal armamentarium, including Mucorales, Fusarium solani and Scedosporium prolificans. In addition, the compound maintained activity versus strains of Candida spp. or Aspergillus spp. that are broadly resistant to azoles, echinocandins or amphotericin B [13,14].

A second effort to target GPI biosynthesis has been recently described and employed chemically induced haploinsufficiency screening to identify small molecule inhibitors of fungal growth [8]. Briefly, the technique used a library of C. albicans heterozygous deletion mutants consisting of about 5400 heterozygote deletion mutants and covering >90% of the fungal genome [15,16]. The response of this mutant library to the presence of sub-MIC concentrations of inhibitor candidate compounds was then characterized by an abundance assay employing DNA microarray analysis. This analysis yielded the relative abundance of specific mutants after compound treatment, and reflects the sensitivity or insensitivity of a mutant to the molecule tested. Although this screen did not specifically target the GPI pathway, small molecules were identified that targeted Gwt1 (G884 and G365) as well as a second GPI pathway enzyme, Mcd4 (M743 and M720) [8]. Mcd4 was previously characterized as an ethanolamine phosphotransferase [7]. Interestingly, the Mcd4 inhibitors, a novel natural product scaffold (M743) as well as a semisynthetic derivative (M720) resulting from the screening revealed a broader spectrum of antifungal activity compared with the Gwt1 inhibitors tested. However, the Gwt1 inhibitors showed lower cytotoxicity relative to the Mcd4 inhibitors, likely because the identified Mcd4 inhibitors also exhibit inhibition of GPI biosynthesis in mammalian cells [17]. Nonetheless, due to the broad spectrum in vitro activity of the Mcd4 inhibitors as well as demonstrated in vivo efficacy without signs of acute toxicity in a murine infection model of candidiasis, Mcd4 appears to merit further exploration to identify potent inhibitors that have improved selectivity toward fungal cells versus mammalian cells.

“...the promise of GPI biosynthesis inhibitors in treating fungal infections is their demonstrated synergy with echinocandins.”

Another recent report identified the small molecule, gepinacin, as a Gwt1 inhibitor [18], originally selected as a false positive from a screen for heat shock protein inhibition. Gepinacin exhibits broad-spectrum antifungal activity, albeit at higher concentrations than E1210 or Mcd4 inhibitors. Interestingly, the report also highlights the potential of Gwt1 inhibitors in enabling recognition of fungi by the immune system [18]. The immune system has multiple pathways for mounting a response to fungal infections including complement, antibody and innate immune Toll-like receptors [19]. Notably, Dectin-1 serves as a pattern recognition receptor that recognizes the pathogen-associated ligand β-(1,3)-glucan within the fungal cell wall. However, fungal cells naturally evade this strategy of host immune recognition via the formation of an outer GPI-derived mannoprotein layer at the cell surface, effectively masking the presence of their underlying β-(1,3)-glucan within the cell wall [19]. Candida albicans treated with sub-MIC levels of any of the above-described GPI inhibitors exposes this underlying β-(1,3)-glucan [8,18]. Consequently, GPI inhibitor-treated C. albicans incubated with a macrophage cell line results in a marked increase in the secretion of TNF-α, an important cytokine associated with systemic inflammatory responses [8,18]. Notably, the enhanced immune system recognition provoked by gepinacin persisted in the presence of both the yeast (nonfilamentous) and hyphal (filamentous) forms of C. albicans [18]. In contrast, echinocandins are able to provoke TNF-α secretion only in the hyphal form of C. albicans [19]. Although the clinical implications of these observations are unknown, the potential for Gwt1 inhibitors to enable better immune recognition of pathogenic fungi is intriguing.

An additional aspect of the promise of GPI biosynthesis inhibitors in treating fungal infections is their demonstrated synergy with echinocandins. In contrast with cancer chemotherapy and antibacterial regimens, combination therapeutic strategies for invasive fungal infections are uncommon. The main exception is the use of fluconazole with amphotericin B for the treatment of cryptococcal meningitis, which has become the standard of care. A primary reason for the dearth of combina-
tion approaches has been the historical demonstration of indifferent or antagonistic interactions between clinically used antifungals such as amphotericin B and azoles or between echinocandins and azoles. In contrast, the Gwt1 inhibitor E1210 has demonstrated in vitro synergy with multiple echinocandins versus numerous strains of A. fumigatus and Aspergillus flavus. In addition, combinations of the two drug classes in vivo have demonstrated efficacy greater than either drug alone. This synergy makes mechanistic sense because in the absence of a coating of GPI-anchored mannanproteins, the β-glucan layer of the cell wall is directly exposed to the echinocandin, affording better access to the echinocandin drug target, β-(1,3)-glucan synthase. For reasons that are not clear, the presence of synergy was less consistent between E1210 and azoles or echinocandins versus different species of Candida. Importantly, there was no observation of antagonism between E1210 and azoles, echinocandins and amphotericin B versus all yeasts and molds tested. Finally, Gwt1 and Mcd4 inhibitors demonstrate strong chemical synergy with each other, paralleling the synthetic lethality of genetic mutations to these targets in S. cerevisiae, suggesting an alternative strategy to consider such inhibitors as novel combination agents.

Although inhibitors of GPI biosynthesis offer potential as broad-spectrum therapeutics, a possible shortcoming is the fungistatic activity of the molecules as described in recent literature reports. However, for E1210, there was a substantial postantifungal effect (PAFE), which is a measurement of how long it takes the test compound-treated fungus to regrow after removal of the test compound for in vitro studies. PAFE was also measured in vivo by assessing regrowth after a single dose of test compound. For C. albicans, the in vitro PAFE was 3.9 h at 16 × MIC (0.13 μg/ml) and 11 h in vivo after a single oral dose of 10 mg/kg in a neutropenic mouse model of invasive candidiasis. For comparison, fluconazole has no significant in vitro PAFE in C. albicans. One rationale for the significant PAFE of E1210 is that the target, Gwt1, is part of a multistep, essential pathway in producing GPI anchors, and this defect in the pathway stresses the endoplasmic reticulum. For example, GPI-anchored protein Gas1 is normally transported to the outer cell wall in yeast. In the presence of gepinacin, the Gas1 precursor accumulates in the endoplasmic reticulum at unusually high levels. In addition, gepinacin induces a considerable unfolded protein response and this also creates stress on the fungi. Therefore, the observed PAFE may be due to the time required for Gwt1 inhibitor-treated fungal cells to recover from these defects in protein trafficking and cellular stress responses.

In summary, targeting GPI biosynthesis may offer considerable potential to develop novel, broad-spectrum antifungal therapeutics. The challenge of developing these GPI targets has likely stemmed from the lack of 3D structural elucidation of the component proteins. In the case of both Gwt1 and Mcd4, a structural challenge is posed by their multiple transmembrane topologies, thereby complicating a protein crystallization effort to assist the rational design of more potent and specific leads. Despite this challenge, multiple groups have discovered highly potent, selective hit and lead compound inhibitors of these targets. Indeed, in the case of Gwt1, cognate inhibitors display potent broad-spectrum antifungal activity lacking appreciable cross-activity against its closest human ortholog, PIG-W. The activity of Gwt1 inhibitors as well as Mcd4 inhibitors versus a broad range of yeast and molds and lack of observed cross-resistance with existing therapeutics should encourage further investigation for novel antifungal leads targeting GPI biosynthesis.

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References
The GPI anchor pathway: a promising antifungal target?  

Commentary


How can we bolster the antifungal drug discovery pipeline?

“Vaccines with antifungal effects seem to hold significant promise for the future.”

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Keywords: antifungals • combination • drug design and development • fungicides • mycoses • nanoparticles • resistance

Fungi are simple eukaryotic organisms that have colonized diverse environments around the planet. There are approximately 2 million different species of fungi on the Earth [1]. Their coexistence with other organisms can vary between mutually beneficial mutualism, commensalism and parasitism. Higher fungi have a long history of use in national cuisines, brewery, viticulture and folk medicine. Investigations of isolated secondary metabolites of higher fungi as well as microfungi have resulted in the discovery of bioactive compounds as potential lead structures for the subsequent design and development of new drugs and other biologically active agents. Yeasts and other fungal species are also used in biotechnology for production or biotransformation of various agents used in medicine [2] as well as for synthesis of nanoparticles within green nanotechnology [3].

Fungi are ubiquitous in nature and vital for recycling of nutrients contained in organic matter. The vast majority of the known fungal species is strict saprophytes, and based on the above-mentioned facts they are very useful. However, some of them can attack humans, animals and plants; it is estimated that 270,000 fungal species are associated with plants, and 325 are known to infect humans [1,4]. Fungi are part of the skin and mucosal microflora of each person. Nevertheless, they can cause diseases varying from unpleasant superficial and cutaneous forms via subcutaneous complicated forms to systemic frequently fatal diseases. Diseases caused by colonization, proliferation and sporulation of fungi in tissues or body fluids are known as mycoses, and invasive fungal infections are the most fatal [5].

It is estimated that about 1.2 million people worldwide suffer from fungal diseases, but a substantial part of these infections are invasive or chronic, and such fungal infections are difficult to diagnose and treat. Annually, 1.5 to 2 million people die from fungal infections worldwide, which is more than from malaria or tuberculosis. Genera Candida (C. albicans, parapsilosis, krusei, glabrata, tropicalis) and Malassezia furfur cause mycoses the most frequently from yeast micro-organisms. C. glabrata, the second most frequently isolated Candida in the EU (>10%) and in the USA (>20%) in the last decade, represents a risk due to its high resistance to fluconazole, voriconazole and echinocandins. Filamentous fungi, such as Trichophyton, Epidermophyton and Microsporum spp. are the most frequent etiological species of superficial mycoses [1,6].

Invasive fungal infections constitute life-threatening diseases especially for immunocompromised population, in other words, patients with AIDS, diabetes mellitus, epidermal and skin lesions, burns, malnutrition, patients undergoing anticancer chemotherapy, transplantation, long-term corticoid therapy or wide-spectrum antibacterial chemotherapeutics. There is also a danger of...
development of invasive fungal infections in case of surgery, catheterization, hemodialysis and parenteral nutrition patients. Very old age and preterm birth are risk factors. The most frequent fungal pathogens causing high mortality are Candida, Aspergillus, Fusarium, Cryptococcus and Pneumocystis. The emerging global threat is the increasing resistance of these species to clinically used antifungals; even cross-resistant, multidrug- or totally resistant strains to all clinically used antifungals can be found and constitute a serious problem [5,6].

Approved antifungal drugs & their new analogues

Human fungal infections generally receive less attention than viral or bacterial diseases; however, mortality from invasive fungal infections is very high, often exceeding 50%. Antifungals are drugs that destroy or prevent the growth of fungi (yeasts, moulds). They can be divided into two main classes: nonspecific antifungals, and site-specific antifungals. Nonspecific antifungals (disinfectants and antiseptics) are applied for superficial/local treatment of skin or mucosa. Although the first specific antimycotic was approved for superficial/local treatment of skin or mucosa, in the 1950s, and since then there has been a significant increase in the number of antifungal drugs used in clinical practice, the treatment of invasive fungal infections is restricted due to the limited number of systemically administered drugs [1,6,7].

“The emergence of antifungal drug resistance calls for the urgency to design new antifungals.”

Clinically used site-specific antifungal drugs can be classified according to the mode of action and their chemical structure as follows: drugs affecting ergosterol, drugs interacting with cell wall (glucan synthesis inhibitors – echinocandins, pneumocandins), inhibitors of transport processes (ciclopirox) and inhibitors of nucleic acid synthesis (flucytosine), protein synthesis (tavaborole) and microtubules synthesis (griseofulvin). Most of the drugs target and inhibit ergosterol-synthesizing enzymes (azoles, thiocarbamates, naphthylmethylamines, phenylpropylmorphanolines) or bind to ergosterol in the cell membrane (polyene antimycotics) [7,8]. Unfortunately, most of drugs have been approved for the treatment of mycoses of nails, skin and mucosa especially due to their narrow therapeutic window, limited bioavailability and drug resistance. Only 5 triazoles (fluconazole, isavuconazole, itraconazole, posaconazole, voriconazole), 3 echinocandins (anidulafungin, caspofungin, micafungin), 1 polyene macrolide (amphotericin B), 1 naphthylmethylamine (terbinafine), 1 pyrimidine (flucytosine) and 1 benzofuran (griseofulvin) have been approved for the treatment of systemic fungal infections [8].

Some new antifungals have been recently approved for treatment of human mycoses, or have been in clinical trials, for example, new azoles (lanosterol 14-α-demethylethyl inhibitors) such as dapaconazole (Zilt® [Biolab Sanus Farmaceutica, Sao Paulo, Brazil]), efinaconazole (Jublia® [ Valeant Pharmaceuticals, Quebec, Canada]), luliconazole (Luzu® [Medicis Pharmaceuticals, AZ, USA]), Lulicon® [Pola Pharma, Tokyo, Japan]) [8], albaconazole (UR-9825), VT-1161 [9] and VT-1129 — a promising candidate for systemic therapy [10]. Also new glucan synthesis inhibitors ASP9726 and biafungin (CD101) from the class of echinocandins have been subject to clinical trials [9]. An orally active derivative of triazole enfumafungin, SCY-078 (MK-3118), is at the early stage of clinical development. Although it possesses the same mechanism of action as other echinocandins, it demonstrates enhanced efficacy for most echinocandin-resistant isolates of C. albicans and C. glabrata as well as for Aspergillus spp. [9]. It is important to note that also 5-[4-(sulfonyl)piperazin-1-yl]-2-arylyrazidin-3(2H)-ones (SCH A–D) and 1-pyrrolidinyl-pyridobenzimidazole-4-carbonitriles (D11-2040, D21-6076) [11] are investigated as new nonechinocandin β-(1,3)- and β-(1,6)-D-glucan synthesis inhibitors.

Emerging resistance

Mechanisms of resistance to antifungals differ among groups of drugs mainly due to the mode of action of each class of antifungals; however, in general resistance can be classified as follows: natural resistance (microorganisms lack the target structure of the drug, and all isolates of the species are resistant; e.g., resistance of some nonalbicans Candida sp. to azoles, amphotericin B); acquired resistance (micro-organisms obtain the ability to resist the activity of the drug to which it was previously susceptible); clinical resistance (therapeutic failure caused by drug pharmacokinetics, drug–drug interactions, patient immunity). The acquired resistance is the most serious. This can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes or from a combination of these two mechanisms [5]. Mechanisms of antifungal resistance can be classified as follows: changes in antifungal transport – in other words, a decrease of effective drug concentration (efflux pumps overexpression or influx decrease; resistance to azoles, naphthylmethylamines and flucytosine); changes in the target structure (enzyme alterations or deficiency/overproduction of some structural components; resistance to azoles, polyenes, echinocandins and naphthylmethylamines);
the use of compensatory mechanisms (metabolic enzyme alterations, metabolic bypasses, toxic-product tolerance; resistance to azoles, polyenes, flucytosine); biofilm formation (complex mechanisms resulting in total changes of micro-organism properties; resistance to azoles, polyenes) [5,12].

Changes in transport inside/outside the cell are the most frequent mechanism of resistance. Influx conditioned resistance is caused by facilitated diffusion of drugs to fungal cells and changes in the composition of the membrane. Drug efflux is connected with over-expression of transport proteins that are able to bind structurally and functionally unrelated compounds, and their stimulation can cause development of multidrug resistance or cross-resistance. Both well-known ATP binding cassette and major facilitator superfamily transporters can be found in the fungal cell membrane. Thus, considerable attention is devoted to understand their structure, function and regulation in order to design/find blockers/modulators of these transporters and sensitize again resistant strains to clinically used drugs [12].

New trends in design of antifungal drugs
The emergence of antifungal drug resistance calls for the urgency to design new antifungals. The discovery procedure of agents with a new mode of action is relatively long and risky; therefore, the approach of the first-choice can be preparation of nanoparticles/nanoformulations of existing drugs. Nanomaterials represent a noteworthy alternative for treatment and mitigation of infections caused by resistant pathogens, which are unlikely to develop resistance to nanomaterials. In contrast to conventional drugs, nanomaterials exert efficiency through various mechanisms; in addition to the drug activity itself, they show ‘intrinsic effects’, such as damaging membrane morphology, disruption of transmembrane energy metabolism and the membrane electron transport chain, generation of reactive oxygen species, etc. In addition, application of nanoformulations enhances the bioavailability of active substances (specific nanoformulations also provide a controlled release system or targeted biodistribution), and the route of administration can be modified. An increase of the efficacy of individual agents can be also ensured by fixed-dose drug combinations or antifungal-active matrices. The US FDA approved some nanoformulations of amphotericin B, for example, Abelcet® (Cephalon Ltd., Harlow, UK), AmBisome® (Gilead Sciences Inc., Uxbridge, UK; Astellas Pharma, IL, USA), Amphoclear® (Three Rivers Pharmaceuticals LLC, PA, USA; Kadmon Pharmaceuticals LLC, NY, USA) and Fungizone® (Bristol-Myers Squibb, NY, USA). Nanoformulations of other antifungal drugs or antifungals conjugated with metal or metal oxide nanoparticles for reinforcement of their effect have been investigated. In addition, nanoformulations of silver, gold, copper, iron or zinc have been extensively tested [13].

A different approach consists in combining antifungal drugs, especially azoles and amphotericin B, with other known drugs or newly designed molecules (e.g., aminoglycosides, antiprotozoals, antipsychotics, calcium channel blockers, berberine, milbemycin, calcineurin inhibitors, HSP90 inhibitors, etc.) that together demonstrate synergistic antifungal properties. These synergistic combinations help to enhance or restore antifungal efficiency of drugs against resistant fungal strains. The main mechanisms of these synergistic effects seem to be perturbation of membrane, disturbance of intracellular ion homeostasis, inhibition of efflux pumps, inhibition of the activity of enzymes required for fungal survival and biofilm formation inhibition [14–19].

The most valuable approach to drug R&D is a rational design of new entities from new chemical classes influencing new targets, in other words, with new mechanisms of action. However, this process is the most expensive, lengthy and uncertain as to the outcome. Also design of new entities from new chemical classes influencing known targets or modification of known entities to impact new targets can be used, although it can be stated that drugs obtained by these two approaches have limited possibilities to solve this critical situation with increasing resistance. One of the reasons, why the R&D process of new antifungals is so complex, is the fact that the eukaryotic nature of a fungal cell is very similar to that of a human cell. Therefore, it is very important to search for antifungal agents, the mechanism of action of which targets the specific structure of the fungal cell.

“The mechanism of action of the majority of existing antifungals used in clinical practice is associated with the cell wall.”

Pharmaceutical industry is closely connected with agrochemical, especially pesticide producers. Some classes of new modern agricultural fungicides can be used for design of structurally new antifungal drugs, since many of them meet criteria of lead-likeliness and/or drug-likeliness, such as the Lipinski’s Rule of Five and the Carr’s Rule of Three. Most newly marketed fungicides have physicochemical parameters within the lead-like range for drugs. In addition, modern fungicides target active sites with high specificity and affinity (in nanomolar or lower concentrations); they are subjected to lead optimization and thus fulfill other requirements of lead chemistry such as tractability in structure–activity relationships and lack of reactivity or promiscuous
binding. The similarity of drugs and pesticides was supported by comparison of the frequency of occurrence of structural fragments in the two types of compounds. Extensive toxicology evaluation, including mammalian toxicology assays, is routinely performed during the whole discovery and development process [7]. Commercial agricultural fungicides are classified according to their target sites by the international Fungicide Resistance Action Committee (FRAC) [20]. From the FRAC classification, it is evident that fungicides show much higher diversity in their chemical structures and modes of action than antifungals. Also as in the case of antibiotic drugs, among agricultural fungicides inorganic, organometallic or organic site-specific fungicides or multi-site fungicides can be found. The most interesting groups of modern specific-target fungicides that could accelerate the process of identification of new modes of action and leads/lead-like structures for a pharmaceutical pipeline to control human fungal pathogens include, for example, inhibitors of adenosin-deaminase, cellulose synthase, 3-keto reductase within ergosterol biosynthesis, DNA/RNA synthesis, trehalase- and inositol-synthase, 3-keto reductase within ergosterol biosynthesis as well as inhibitors of adenosin-deaminase, cellulose synthase, 3-keto reductase within ergosterol biosynthesis, DNA/RNA synthesis, trehalase- and inositol-synthase, 3-keto reductase within ergosterol biosynthesis.

The mechanism of action of the majority of existing antifungals used in clinical practice is associated with the cell wall. It remains a major objective for the development of other potential antifungals, because it contains components specific for fungal cells. Another possible way in the development consists in the inhibition of transition to the fibrous form by dimorphic pathogens, which leads to a reversible change from a saprophytic to a pathogenic form. Potential antifungal active compounds are also host defense peptides and cationic antimicrobial peptides due to the low risk of resistance to them. New targets can also include agents affecting biosynthesis of chitin, glycosylphosphatidylinositol, glucosylceramide, heme; affecting virulence and mitochondrial functions, generating of oxygen radicals; or inhibiting dihydroorotate dehydrogenase.

Renewed nikkomycin Z, a competitive chitin synthase inhibitor lacking mammalian toxicity with effect against Coccioidoides spp. is under clinical trial [9]. E1210 (3-[isoxazol-5-yl]pyridin-2-amine derivative) is an orally active broad-spectrum inositol acyltransferase inhibitor with high potency against Candida, Aspergillus and Fusarium suitable for treatment of disseminated candidiasis and pulmonary aspergillosis [11]. Glycosylphosphatidylinositol inhibitors include Gwt1 inhibitors (e.g., gepinacin [G642], G365 and G884 with an effect against C. albicans and A. fumigatus) and Mcd4 inhibitors (e.g., M720 with tetradecahydroindeno[5′,6′:4,5]cycloocta[1,2-c]pyran-2[1H]-one scaffold and potency against Cryptococcus neoformans and Pneumocystis jiroveci [21]. Compound SM21 (2,6-diterter-butyl-4-[(E)-2-[4-(dimethylamino)phenyl]ethenyl]pyranium) with effect against multidrug-resistant Candida spp. and Candida biofilms is an agent influencing virulence, which inhibits change morphology between yeast and filamentous forms [11]. Ilicicinol H (4-hydroxyppyridin-2[1H]-one derivative) isolated from Gliocadium roseum, an inhibitor of mitochondrial cytochrome bc1 reductase, with activity against Candida spp., A. fumigatus, and Cryptococcus spp. is limited in application due to high plasma protein binding [23]. On the other hand, hydroxyarylpyrazoles, for example, ME1111, were discovered as selective inhibitors of succinate-coenzyme Q reductase (or respiratory Complex II) of Trichophyton sp. [9]. Functional static arylamide T-2307 showed activity against Candida spp., A. fumigatus, and Aspergillus spp. This compound causes a collapse of the mitochondrial membrane potential and is in the 1st phase of clinical trials [9]. Thioene derivative of sampagine, an alkaloid isolated from Cananga odorata inhibiting generation of heme and initiating production of free oxygen radicals, showed high activity against A. fumigatus and C. neoformans [24]. F901318 from the group of orotomides – compounds with unique mode of action – is dihydroorotate dehydrogenase (essential enzyme for pyrimidine synthesis) inhibitor in the first Phase of clinical trials that demonstrates significant activity against Aspergillus spp. [9]. Compound VL-2397 (ASP2397) is a new intravenously administered fungicidal antifungal with an unknown mechanism of action. The siderophore-mediated uptake of VL-2397 to fungal cells causes high selectivity of this compound. It was isolated from Acremonium spp. and shows excellent efficiency against multidrug resistant A. fumigatus and C. glabrata. The first Phase of clinical trials is being prepared [10].

Antimicrobial peptides may be considered as interesting therapeutic agents. They are cationic endogenous polypeptides produced by metazoans and causing membrane lysis of negatively charged surface microbial membranes. They are effective against multidrug resistant pathogens and do not have any potential for development of resistance, nevertheless due to their instability, limited bioavailability and high price they have not been registered so far. The following antimicrobial peptides effective especially against Candida sp. and Aspergillus...
spp. are in the second Phase of clinical trials: (CKPV) peptide (CZEN-002), lactoferin 1–11 (hLF1–11), PAC113 (P1-113), NP339/NP525 (Novamycin) [9].

Vaccines with antifungal effects seem to hold significant promise for the future. They could be useful for prevention and a decrease of morbidity and mortality and can help to reduce societal costs. Possible indications include various candidiases of ordinary as well as immunosuppressed patients. Prophylactic recombinant vaccines such as NDV-3, PEV-7 and rHyr1p-N are in clinical trials [9].

In conclusion, beside the design of structurally new antifungals based on new targets (single- or multi-site antifungal agents), promising strategies to combat antifungal drug resistance seem to be the design of efflux inhibitors, various chemosensitizers, inhibitors of pH signaling pathways, biofilm formation, filamentation and virulence as well as genome-wide studies.

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References
Strategies in the discovery of novel antifungal scaffolds

The development of next-generation antifungal agents with novel chemical scaffolds and new mechanisms of action is vital due to increased incidence and mortality of invasive fungal infections and severe drug resistance. This review will summarize current strategies to discover novel antifungal scaffolds. In particular, high-throughput screening, drug repurposing, antifungal natural products and new antifungal targets are focused on. New scaffolds with validated antifungal activity, their discovery and optimization process as well as structure–activity relationships are discussed in detail. Perspectives that could inspire future antifungal drug discovery are provided.

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Keywords: antifungal drug resistance • antifungal lead compounds • drug repurposing • fungal biofilm inhibitors • invasive fungal infections • new antifungal scaffolds • strategies in antifungal drug discovery

It is estimated that there are approximately 600 species of human fungal pathogens. Among them, approximately 20 species cause more than 99% of human fungal infections, which can be classified into superficial fungal infection and invasive fungal infection (IFI). IFIs are often life-threatening and associate with high mortality in immunocompromised hosts such as patients with AIDS and patients undergoing organ transplants or anticancer chemotherapy [1,2]. *Candida albicans* (mortality rate: 20–40%), *Cryptococcus neoformans* (mortality rate: 20–70%), and *Aspergillus fumigatus* (mortality rate: 50–90%) are the most common pathogens of IFIs [3,4].

Clinically, antifungal agents for the treatment of life-threatening IFIs (Figure 1) mainly include amphotericin B (AmB), azoles (e.g., fluconazole, itraconazole and voriconazole), echinocandins (e.g., caspofungin, micafungin and anidulafungin) and 5-fluorocytosine (usually used as adjunctive therapy) [5,6]. AmB is a polyene antibiotic that targets on fungal cell membrane by selective interaction with ergosterol [7]. Although AmB is still considered as the ‘gold standard’ for some severe infections [8], it has serious nephrotoxicity and many other side effects [9]. Extensive efforts have been made to reduce the toxicity of AmB including the development of lipid formulations and synthesis of new derivatives [7,10]. Azole antifungal agents are inhibitions of CYP51 in fungal cell membrane and are widely used as the first line of antifungal therapy [11]. However, their antifungal potency has been significantly reduced because of severe drug resistance [12–14]. Development of new triazole is still an active area. For example, isavuconazole has been approved by the US FDA in 2015 for treatment of invasive aspergillosis and invasive mucormycosis [15] in adults. Echinocandins are the newest class of antifungal agents, which act by inhibition of GS in fungal cell wall [16]. Although echinocandins are fungicidal with good selectivity, they cannot be orally administrated because of their complex lipopeptide structures [17]. To address the limitation, several small molecule GS inhibitors have been discovered [18]. However, none of them is under clinical evaluation.
In recent years, the incidence and mortality of IFIs has been increasing dramatically due to the increasing number of immunocompromised hosts and limitations of current antifungal therapy. First, clinically available antifungal agents are very limited and far from satisfaction [19]. New drugs are expected to
overcome their drawbacks, such as limited-potency, narrow-spectrum, nonoptimal pharmacokinetics, drug-related toxicity and drug–drug interactions. Second, drug resistance has been observed for all of the three classes of antifungal agents [20]. Although several new triazoles (e.g., albaconazole [21]) and echinocandins (e.g., aminocandin [2]) are under clinical or preclinical trials, these ‘me too’ drugs with the same mechanism of action cannot solve the resistance problem fundamentally. Third, infections caused by new fungal pathogens, such as hyaline moulds (Fusarium and Scedosporium), zygomycetes as well as opportunistic yeast-like fungi (Trichosporon and Rhodotorula), are also increasing in recent years. They are generally more difficult to diagnose and treat and are associated with greater mortality. Therefore, there is an emergent need to discover and develop new generation of antifungal agents with novel chemical scaffolds and fungal-specific mode of action.

An ideal antifungal agent should have fungicidal activity, good selectivity toward a fungal-specific target, a broad-spectrum, low risk to develop resistance, reasonable pharmacokinetic (PK) and pharmacodynamic (PD) profile, and low toxicity or side effects. They could serve as guidelines for discovery and development of new generation of antifungal agents. However, the progress in antifungal drug development is relatively slow, particularly lagging behind the development of antibacterial agents. The main difficulty is attributed to the eukaryotic nature of fungal cells, which share high similarity with human counterparts. Thus, the discovery of new chemical scaffolds acting on fungal-specific targets is highly desirable.

In 2011, new lead compounds in antifungal drug discovery were reviewed by our group [22]. Herein we will primarily focus on current strategies to discover novel antifungal scaffolds. Moreover, examples of antifungal lead compounds were mainly selected from recent publications (2011–2015). In particular, new antifungal scaffolds whose antifungal activity has been fully validated by different assays and/or a combination of in vitro and in vivo models will be discussed in detail.

**Screening-based strategies to discover novel antifungal scaffolds**

Historically, the most common approach for antifungal drug discovery is to screen large compound libraries (synthetic small molecules or natural products). Clinically available antifungal agents, namely AmB, triazoles and echinocandins, were all originally discovered by screening for their ability to inhibit the growth of fungal pathogens prior to know their mode of action. In recent years, phenotypic screening continues to be a major approach to discover novel antifungal scaffolds. This section will mainly focus on screening methods and antifungal scaffolds derived from libraries of synthetic small molecules. Screening of natural products will be discussed in the ‘Natural product-based strategies to discover novel antifungal scaffolds’ section.

**Compound library for antifungal screening**

The efficiency of screening-based antifungal drug discovery largely depends on the quality of compound library and antifungal assay. Recently, the number of compound libraries has been increasing dramatically, which offers opportunities for novel antifungal drug discovery. These compound libraries are commercially available (e.g., SPCES, ChemDiv, Maybridge), freely accessed (e.g., NIH Libraries Program) or privately owned (in-house libraries of pharmaceutical companies or research groups). Structural diversity (particularly scaffold diversity) and drug-likeness (favorable physicochemical properties) are widely regarded to be the main characteristics of a high-quality compound library. Highly efficient synthetic methods, such as diversity oriented synthesis (DOS) [23,24], were proven to be fruitful tools to construct high-quality compound libraries and provide a valuable source of biologically active lead compounds. For example, our group reported a new synthetic method, divergent organocatalytic cascade approach (DOCA), to build compound libraries with good scaffold diversity and drug-likeness [25]. The DOCA-derived library was assayed for antifungal activities and two compounds (10 and 11) displayed antifungal activities against *C. albicans* and *C. neoformans* with minimum inhibitory concentration (MIC) values ranging from 8 μg/ml to 32 μg/ml (Figure 2) [25]. Notably, their scaffolds are totally different from the reported antifungal compounds.

**New antifungal scaffolds derived from traditional assays**

Currently, there are two standards, the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), for in vitro susceptibility testing of the selected fungal pathogens [26,27]. Both of them use traditional broth or microbroth growth inhibition method and measure the microbial growth by optical density of the culture. The standard assays are well-accepted protocols to evaluate the in vitro antifungal activities. Also, they are effective tools to high-throughput screening of compound libraries. For
example, new 1,2-benzothiazoline (12) [28,29], 2-(2-oxo-morpholin-3-yl)-acetamide (13) [30] scaffold were identified by screening of compound libraries, which showed potent antifungal activities with a broad spectrum (Figure 3).

Our group performed an antifungal screen of an in-house library and identified β-carboline scaffold 14 as a new antifungal agent (Figure 4) [31]. Then, a series of carboline derivatives (15) were designed and synthesized to investigate the structure–activity relationship (SAR). As a result, the optimized compound 16 showed improved antifungal activity and was comparable to fluconazole (Figure 4) [31]. Interestingly, compound 16 showed several promising features as a novel antifungal lead compound. First, it exhibited good fungicidal activity against both fluconazole-sensitive and fluconazole-resistant C. albicans cells [31]. Second, compound 16 was also proven to be a good inhibitor of C. albicans biofilm formation and hyphal growth, highlighting the potential to overcome fluconazole-related and biofilm-related drug resistance [31]. Third, it showed good synergistic activities in combination with fluconazole. Last, compound 16 had weak inhibitory activity against CYP enzymes, indicating that it had low potential to cause drug–drug interactions [31]. Preliminary mechanism studies revealed that the carboline lead compound might act on fungal cell wall [31].

The development of new assays and discovery of new antifungal scaffolds

Limitations of traditional antifungal assays mainly include poor correlation between fungal growth and optical density for some species (e.g., Aspergillus) and inability to identify fungal biofilms inhibitors. Moreover, they are unable to distinguish between molecules with fungistatic and fungicidal activities. Recently, a number of antifungal screening assays were reported to address the limitations of traditional methods [32]. For example, adenylyl kinase-based [33] and alamarBlue-based [34] high-throughput screening assays have been developed to the specific discovery of fungidical agents. Rabjohns et al. screened 1280 small-molecule compounds using the alamarBlue-based assay and identified a potential rhodanine scaffold (17) that exhibited fungidical activity in the low micromolar range (Figure 5) [34]. Compound 17 showed good fungidical activity against C. neoformans with low toxicity. However, it needs further structural optimization to exert efficacy in the mouse cryptococcal meningitis model because of its low half-life ($t_{1/2} = 10$ min) in mice.

There are three different morphological forms (i.e., yeast, pseudohyphae and hyphae) of Candida cells. During the fungal infection, the first step is adhesion to surfaces, and a subsequent yeast-to-hyphal morphology transition is a major virulence that leads to tissue invasion and biofilm formation. The strong ability of Candida cells to form biofilms is an important reason for the emergence of high levels of resistance to most clinically used antifungal agents [35–38]. Thus, fungal biofilm inhibitors have low potential for the development of resistance and represent a new area to develop novel antifungal therapies [39]. XTT and alamarBlue-based assays using metabolic activity as reporters were developed for high-throughput screening against C. albicans biofilms [40,41]. For example, the alamarBlue-based assay was successfully used to identify 1,3-benzothiazole scaffold (18) as potent C. albicans biofilm inhibitors [40]. Another high-throughput screening discovered the diazaspire-decane scaffold (19) to be potent inhibitors of biofilm formation and filamentation [42]. Compound 19 showed good inhibitory activity against C. albicans biofilms (IC$_{50} = 2.7\, \mu$M).
with low toxicity (CC$_{50}$ = 104.1 μM). Moreover, it inhibited C. albicans filamentation without affecting overall growth under planktonic conditions. Interestingly, resistance was not developed after repeated exposure to compound 19. The lead compound also showed in vivo efficacy in clinically relevant murine models of invasive and oral candidiasis.

Moreover, new assays for high-throughput screening of adhesion [43] and yeast-to-hyphal [44] inhibitors were also developed. Fazly et al. screened a library of 30,000 small molecules and identified the 1-benzoyl-4-phenylpiperazine scaffold that inhibited adhesion of C. albicans to cultured human epithelial cells [43]. The lead compound (named filastatin, 20) could effectively inhibit C. albicans biofilm formation and hyphal morphogenesis. Consistent with its in vitro activities, filastatin showed in vivo antifungal activity in a nematode model of C. albicans infection. More recently, Wong et al. identified a series of inhibitors of yeast-to-hypha transition from a collection of over 50,240 small molecules. Among the hits, SM21 (21) bearing a 4-(dimethylamino)styrlypyrylium scaffold exhibited highly potent antifungal activity both in vitro and in vivo [44]. The MICs of SM21 against the Candida
and other fungal species ranged from 0.2–6.25 μg/ml. Moreover, it was still active against resistant *Candida* spp. with MIC values in the range of 0.5–1 μg/ml. Besides inhibition of yeast-to-hypha transition, SM21 also inhibited biofilm formation and displayed low toxicity to human cells. In the oral candidiasis mouse model, oral rinses containing 200 μg/ml SM21 could significantly reduce tongue lesions, whose efficacy of SM21 was better than that of nystatin.

Besides the in vitro assays, Mylonakis’s group developed a whole-animal high-throughput antifungal assay using *Caenorhabditis elegans* as the model hosts [45,46]. The *Candida*-mediated *C. elegans* assay allows high-throughput in vivo screening of compound libraries for both antifungal activities and toxicity. The in vivo assay has been used to identify several new antifungal lead compounds. Among them, caffeic acid phenethyl ester (CAPE, 22) exhibited antifungal activity in a murine model of candidiasis [45].

**Drug repurposing-based strategies to discover novel antifungal scaffolds**

The concept of ‘drug repurposing’ is the application of known drugs to new diseases, which has emerged as an promising approach to accelerate drug development [47]. As compared with traditional drug development, a significant advantage of drug repositioning is that the toxicology and pharmacology of the marked drugs have already been well-established. As a result, the risk of failure for development of an old drug with a useful new indication could be significantly reduced. Several studies have identified a number of ‘nonantifungal’ drugs with antifungal activity by screening libraries of marketed drugs [48]. The antifungal activity and/or synergic effects with fluconazole of rapamycin [49], sertraline [50] and tamoxifen [51] have been reviewed by Krysan’s group [48]. The same group also screened the Prestwick library of 1,120 off-patent drugs to identify drugs with fungicidal activity toward *C. neoformans* [52]. A total of 31 hits were detected and 15 of them were newly identified. A large portion of the hits shares common structural features, namely a hydrophobic moiety (labeled red in Figure 6) linked to a basic amine (labeled blue in Figure 6) [52]. Another interesting feature of them is the ability of crossing the blood–brain barrier, highlighting their potential application as anticryptococcal agents. Two repre-
Figure 8. Chemical structures and antifungal activity of seven newly identified antifungal scaffolds derived from marketed drugs.

MIC0.3: Minimal concentration of drug resulting in 30% growth inhibition.

Amonafide (28) Antineoplastic agent
MIC0.3 = 1.40 μg/ml Antineoplastic agent

Tosedostat (29) Antileukemic agent
MIC0.3 = 4.0 μg/ml

Megestrol acetate (30) Antineoplastic agent
MIC0.3 = 0.39 μg/ml

Melengestrol acetate (31) Antineoplastic agent
MIC0.3 = 0.33 μg/ml

Stanozolol (32) Anemia and hereditary angioedema
MIC0.3 = 0.37 μg/ml

Trifluperidol (33) Antipsychotic agent
MIC0.3 = 0.40 μg/ml

Haloperidol (34) Antipsychotic agent
MIC0.3 = 0.46 μg/ml

Strategies in the discovery of novel antifungal scaffolds

Representative hits, thioridazine (23, MIC = 16 μg/ml) and amiodarone (24, MIC = 8 μg/ml), were active against intraphagocytic *C. neoformans* and were additive or synergistic to fluconazole [52]. Preliminary mechanism studies revealed that they acted by directly binding *C. neoformans* calmodulin [52].

Siles et al. screened Prestwick Chemical Library containing 1200 FDA-approved, off-patent drugs for biofilm inhibitors [53]. Three old drugs, auranofin (anti-inflammatory agent, 25, IC50 = 6.1 μM), benz bromarone (antigout agent, 26, IC50 = 2.0 μM) and pyrvinium pamoate (antihelminthic agent, 27, IC50 = 6.7 μM) were validated as potent inhibitors of *C. albicans* biofilm formation (Figure 7). However, their antifungal efficacy was not reported in this work. Interestingly, another study identified that a combination of pyrvinium pamoate and fluconazole was effective toward azole resistance in *C. albicans* [54].

More recently, Stylianou et al. screened a library of 844 drugs (approved or in clinical trial) for anti-*Candida* activity based on the EUCAST guidelines [55]. Seven drugs, namely amonafide (28), tosedostat (29), megestrol acetate (30), melengestrol acetate (31), stanozolol (32), trifluperidol (33) and haloperidol (34), were newly identified to possess antifungal activity (Figure 8). Their antifungal activities were validated by three individual assays and different *Candida* spp.
Box 1. Selected examples of new antifungal scaffolds from natural products.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>Candida albicans MIC_{50}</th>
<th>Cryptococcus neoformans MIC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Compound 35" /></td>
<td>Hyalis argentea var. latisquama</td>
<td>15.6 μg/ml</td>
<td>15.6 μg/ml</td>
</tr>
<tr>
<td><img src="image" alt="Compound 36" /></td>
<td>Guignardia sp. and Guignardia mangiferae</td>
<td>15.5 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Compound 37" /></td>
<td>Guignardia sp.</td>
<td>87.5 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Compound 38" /></td>
<td>Guignardia sp. and Coniothyrium sp.</td>
<td>24.3 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Compound 39" /></td>
<td>Alternaria alternate</td>
<td>13.7 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Compound 40" /></td>
<td>Alternaria alternate</td>
<td>17.1 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Compound 41" /></td>
<td>Alternaria alternate</td>
<td>32.0 ± 2.1 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="Compound 42" /></td>
<td>Sagittaria latifolia</td>
<td>9.2 μg/ml</td>
<td></td>
</tr>
</tbody>
</table>

**Box 1. Selected examples of new antifungal scaffolds from natural products (cont.).**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>Trichophyton rubrum MIC_{80}</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Compound 41" /></td>
<td>Alternaria alternate</td>
<td>32.0 ± 2.1 μg/ml</td>
</tr>
</tbody>
</table>

**References:**

[60, 61, 62, 63, 64, 65, 66, 67, 68]
Box 1. Selected examples of new antifungal scaffolds from natural products (cont.).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>C. albicans MIC</th>
<th>C. neoformans MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="43" /></td>
<td>Swartzia simplex</td>
<td>32 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image2" alt="44" /></td>
<td>Swartzia simplex</td>
<td>32 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image3" alt="45" /></td>
<td>Pseudaxinella reticulata</td>
<td>14 μM</td>
<td>0.85 μM</td>
</tr>
<tr>
<td><img src="image4" alt="46" /></td>
<td>Pseudallescheria boydii SNB-CN73</td>
<td>2 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image5" alt="47" /></td>
<td>Beilschmiedia aloiophylla</td>
<td>8 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image6" alt="48" /></td>
<td>Fimetariella sp.</td>
<td>5 μg/ml</td>
<td></td>
</tr>
<tr>
<td><img src="image7" alt="49" /></td>
<td>Clausena excavata</td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="image8" alt="50" /></td>
<td>Dimocarpus longan Lour.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
clinical isolates. The results showed that the antifungal activities of these drugs were comparable to those of the clinically available antifungal agents (e.g., fluconazole, amorolfine). Moreover, the antifungal activity of auranofin that was previously identified as a biofilm inhibitor was validated in this study (C. albicans, MIC = 0.68 μg/ml). Up to now, about 20 old drugs were reported to have antifungal activity, which could serve as pharmacologically attractive scaffolds for further development. However, in-depth evaluation and particularly structural optimization of them is still rare, which limited their therapeutic application as novel antifungal agents. Subsequent work should aim to optimize their antifungal properties and reduce their original functions.

Natural product-based strategies to discover novel antifungal scaffolds

Natural products are rich sources of novel antifungal agents [56,57]. Polyenes and echinocandins, two major classes of antifungal agents, are originated from natural products [56]. Natural product-based discovery of novel antifungal agents is becoming an active research area due to their structural diversity [22, 57]. Recent examples of antifungal natural products (35–50) are summarized in Box 1. However, the challenges of natural product-based antifungal discovery mainly include the structural and stereo-chemical complexity, difficulty in chemical synthesis and structural modifications, and unfavorable physicochemical properties for drug development [58]. To tackle these problems, substitution optimization and scaffold hopping present a promising strategy to improve the antifungal activity, PK/PD profile of natural products [59]. Moreover, elucidation of the structural requirements for antifungal activity and structural simplification of the natural scaffold are effective methods to discover highly potent but less complex scaffolds. The following section will introduce our efforts in this field.

Scaffold hopping & structural simplification of antifungal natural product sampangine

Sampangine (51), an azaoxoaporphine alkaloid extracted from the stem bark of Cananga odorata, showed broad spectrum antifungal activity against various human fungal pathogens including C. albicans, C. neoformans and Aspergillus fumigatus [76,77]. Although several A- and B-ring-substituted [78] and hetero analogs [79] of sampangine derivatives have been reported, further drug development was hampered because of poor water solubility and lack of in vivo antifungal efficacy. In order to address the problems, we performed a scaffold hopping study of sampangine using heterocycles to replace the D-ring (Figure 9). The thiophene derivative ZG-20 (52) revealed improved activity against C. neoformans (MIC...
Screening of compound library

Optimization

C. albicans MIC = 1.56 μg/ml
A. fumigatus inactive

57
C. albicans MIC = 0.1 μg/ml
A. fumigatus MIC = 1.56 μg/ml

58
C. albicans MIC = 0.05 μg/ml
A. fumigatus MIC = 0.78 μg/ml

59
C. albicans MIC = 0.39 μg/ml
A. fumigatus MIC = 3.13 μg/ml

E-1210 (60)
C. albicans MIC = 0.004 μg/ml
A. fumigatus MIC = 0.03 μg/ml

Figure 10. Chemical structures, structural optimization process and antifungal activity of inhibitors of fungal cell wall GPI biosynthesis.

MIC: Minimum inhibitory concentration.

= 0.25 μg/ml) and A. fumigatus (MIC = 2 μg/ml) and enhanced water solubility (solubility: 48 μg/ml) [80].
Inspired by the results, the simplification of sampangine scaffold was further investigated [81]. Interestingly, A-ring removed analog ZG-20-07 (53, MIC range: 0.125 μg/ml–2 μg/ml) and A, B-ring removed analog ZG-20-41 (54, MIC range: 0.5 μg/ml to 4 μg/ml) showed excellent antifungal activity against a variety of fungal pathogens. Moreover, ZG-20-07 (solubility: 38.6 μg/ml) and ZG-20-41 (solubility: 65.2 μg/ml) showed significantly improved water solubility than sampangine (solubility: 12.6 μg/ml). Interestingly, they exhibited fungicidal activity on both fluconazole-sensitive and fluconazole-resistant C. albicans. In a Caenorhabditis elegans–C. albicans infection model, ZG-20-07 and ZG-20-41 showed good in vivo antifungal activity with low toxicity and could effectively protect the C. albicans infection. In addition, both of them inhibited C. albicans biofilm formation, cellular surface hydrophobicity (CSH) and yeast-to-hypha morphological transition of C. albicans in a dose-dependent manner, highlighting their potential as good lead compounds for the treatment of resistant fungal infections.

Target-based strategies to discover novel antifungal scaffolds

Clinically available antifungal agents mainly target on ergosterol in cell membrane (AmB), CYP51 in fungal cell membrane (azoles) and GS in fungal cell wall (echinocandins). Among them, only the crystal structure of Saccharomyces cerevisiae CYP51 was solved [82]. Our group used computational approaches to structure-based rational design new triazole CYP51 inhibitors and nonazole inhibitors [83,84]. For GS, the discovery of
new inhibitors, particularly small molecule inhibitors, still mainly depends on high-throughput screening [18]. On the other hand, the development of new antifungal drugs based on new targets is highly desirable due to the limitations of current antifungal agents and the emergence of severe resistance [85]. Over the past few years, important progress has been made in fungal genomics, which provide a good opportunity to identify novel antifungal targets [86]. The genomes of important fungal pathogens, such as C. albicans, C. neoformans and A. fumigatus, have been released and functional proteins that are essential for fungal growth are important source of antifungal drug targets. In particular, targets that are conserved among fungal pathogens and lack a human counterpart are highly promising for drug discovery and development. Moreover, a druggable antifungal target should be able to bind drug-like compounds, which needs to be validated by medicinal chemistry efforts.

Currently a variety of new antifungal targets are already available, which can be classified into four types according to their location and functions in fungal cells. They are fungal cell wall targets (e.g., chitin synthase, and mannoprotein), fungal cell membrane targets (e.g., inositol phosphoceramide synthase), DNA and protein synthesis targets (e.g., N-myristoyltransferase, aminoacyl-tRNA synthetase, elongation factor, secreted aspartic proteinase, topoisomerase) and signal transduction pathway targets (e.g., calcineurin, electron transport chain). Most inhibitors acting on these targets have been reviewed by our group and others [22,87–89]. Herein only recent progress of new antifungal targets, new scaffolds as well as discovery and optimization strategies was highlighted.

**GPI inhibitors**

GPI-anchored proteins, a kind of cell wall mannoproteins, are required for the adhesion of pathogenic fungi to human epithelium [90]. Due to the importance of GPI-anchored proteins in fungal cell wall biosynthesis and maintenance of homeostasis, designing inhibitors targeting this biosynthetic pathway has the advantage of reducing adverse effects in human cells. Up to now, there are two scaffolds reported to inhibit fungal GPI biosynthesis (Figure 10) [91,92]. 1-(4-Butylbenzyl)isoquinoline (BIQ, 55) is the first GPI-anchored protein inhibitor discovered by means of a yeast cell-based screening [91]. BIQ has moderate inhibitory activity against C. albicans.
Figure 12. Chemical structures and binding mode of dihydrofolate reductase inhibitors. (A) Chemical structures and antifungal activity of fungal dihydrofolate reductase inhibitors; (B) the binding mode of a 2,4-diaminopyrimidine propargyl inhibitor with Candida albicans dihydrofolate reductase. The figure was generated on the basis of the crystal structure obtained from Protein Data Bank (PDB code: 4HOF). DHFR: Dihydrofolate reductase; IC₅₀: Inhibitory activity of biofilm formation; MIC: Minimum inhibitory concentration.

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>IC₅₀ C. albicans</th>
<th>MIC C. albicans</th>
<th>IC₅₀ C. glabrata</th>
<th>MIC C. glabrata</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>30 nM</td>
<td>&gt;100 ug/ml</td>
<td>28 nM</td>
<td>12.5 ug/ml</td>
</tr>
<tr>
<td>66</td>
<td>20 nM</td>
<td>1.5 ug/ml</td>
<td>5.5 nM</td>
<td>0.39 ug/ml</td>
</tr>
<tr>
<td>67</td>
<td>49 nM</td>
<td>0.39 ug/ml</td>
<td>27 nM</td>
<td>0.2 ug/ml</td>
</tr>
</tbody>
</table>

(C. albicans DHFR IC₅₀ = 30 nM, MIC > 100 ug/ml; C. glabrata DHFR IC₅₀ = 28 nM, MIC = 12.5 ug/ml; C. albicans DHFR IC₅₀ = 20 nM, MIC = 1.5 ug/ml; C. glabrata DHFR IC₅₀ = 5.5 nM, MIC = 0.39 ug/ml; C. albicans DHFR IC₅₀ = 49 nM, MIC = 0.39 ug/ml; C. glabrata DHFR IC₅₀ = 27 nM, MIC = 0.2 ug/ml)

Meta-linked 65
Para-linked 66

AHAS inhibitors
AHAS or ALS is the first enzyme in the biosynthesis of branched-chain amino acids [100]. AHAS is highly conserved in fungi without human counterpart, which is a potential target for the development of selective antifungal agents [88]. In 2013, two scaffolds were identified as fungal AHAS inhibitors by different strategies (Figure 11) [101,102]. Richie et al. used high-throughput phenotypic screening in combination with chemogenomic profiling strategy to discover triazolopyrimidine-sulfonamide scaffold...
as *S. cerevisiae* AHAS inhibitors [102]. Compounds 61 and 62 had broad-spectrum *in vitro* activity, no significant cytotoxicity, and low protein binding. In another study, Lee et al. used *C. albicans* AHAS assay to screen eight commercial sulfonylurea herbicides. Chlorimuron ethyl (63) is the most potent compound in both AHAS inhibition assay (*K*_i = 7 nM) and antifungal activity assay (*C. albicans* MIC = 2 μM). Structural optimization of 63 led to the discovery of the iodine analog 64 with improved activity (*AHAS K*_i = 3.8 nM, *C. albicans* MIC = 0.6 μM). In both studies, the binding mode of inhibitors with AHAS were analyzed on the basis of the crystal structure of *S. cerevisiae* AHAS. However, structure-based design and optimization of AHAS inhibitors have not been reported up to now.

**DHFR inhibitors**

Dihydrofolate reductase (DHFR), a key enzyme in thymidine synthesis, is a traditional antitumor target. In recent years, targeting DHFR has also proven to be an effective strategy in antimicrobial drug discovery. There are several important differences in the active site between human and *Candida* species, which provides basis for the design of selective inhibitors. Although fungal DHFR inhibitors have been reported [103], a major problem is the poor correlation between DHFR inhibitory activity and antifungal activity. Anderson’s group reported a 2,4-diaminopyrimidine propargyl scaffold as *Candida* DHFR inhibitors (Figure 12) [104–106]. Moreover, compounds containing a para-linked biphenyl moiety (66) had a broader spectrum than the corresponding meta-linked compounds (65). The binding modes of this class of inhibitors with *C. albicans* and *C. glabrata* DHFR were confirmed by determining crystal complexes [107]. The pyrimidine ring of the inhibitor forms conserved hydrogen bonding and hydrophobic interactions with Glu32, Ile9, Phe36, Met/Ile53 and Ile121 (Figure 12). The propargyl linker and biphenyl moiety form hydrophobic and van der Waals interactions with DHFR. The crystal structures also revealed that additional hydrophobic functionality substituents to the para position of the distal C-ring may enhance the potency of enzyme inhibition and selectivity alter the physicochemical properties. On the basis of the binding model, a series of new para-linked compounds were designed, most of which inhibited both *C. albicans* and *C. glabrata* DHFR and had dual antifungal activity. For example, compound 67 showed MIC values lower than 0.5 μg/ml against both *C. albicans* and *C. glabrata*.

**Inhibitors of β-1,6-glucan synthesis**

β-1,6-glucan is an important fungal cell wall component [108]. Researchers from Daiichi Sankyo (Japan) developed a cell-based assay for screening inhibitors of
cell wall components and identified the first inhibitor (D75-4590, 68) of β-1,6-glucan synthesis [109]. D75-4590 acted by targeting Kre6p and showed moderate activities against a variety of Candida species including fluconazole-resistant strains, but was inactive toward C. neoformans and Aspergillus species. Subsequently, D75-4590 was optimized to improve the antifungal activity and physicochemical properties (Figure 13). First, medicinal chemistry efforts were focused on the C1-C4 substitutions on pyridobenzoimidazole scaffold [110]. The SAR revealed that the C3-methyl group, C4-cyano group and a terminal amine in C1-side chain were essential for antifungal activity. In contrast, the introduction of hydrophobic group to the C-2 position and and cyclic amine in the C1 side chain enhanced antifungal activity. Compound D11-2040 (69) showed excellent activity against C. glabrata (MIC = 0.016 μg/ml) [110,111].

Using the scaffold hopping strategy, D11-2040 was further optimized by inserting a nitrogen atom in the phenyl part of pyridobenzoimidazole scaffold. The resulting compound D21-6076 (70) had improved physicochemical properties and showed good in vivo efficacy in the C. glabrata infection model [112]. Interestingly, although compound D21-6076 was poorly active to inhibit the growth of C. albicans in the in vitro assay, it still showed protective effects in the C. albicans infection model. Mechanism studies revealed that compound D21-6076 may act by inhibiting the invasion process of C. albicans [112]. To further optimize the water solubility and metabolic stability, a structural simplification strategy was used to design bicyclic derivatives (i.e., triazolopyridines, imidazopyridines, and pyrazolopyridines, 71–73) [113]. After the removal of a phenyl group, the triazolopyridine scaffold (71) showed improved water solubility and retained the excellent activity against C. glabrata [113]. However, in vivo results of these bicyclic scaffolds have not been reported.

Conclusion

In summary, current strategies for the discovery of novel antifungal scaffolds were reviewed. High-throughput screening (HTS) of compound library is still a useful and effective tool to identify new antifungal compounds. However, the hit rate of HTS is relatively low. Drug repurposing is a special kind of HTS using marketed drugs as compound library. As compared with traditional HTS hits, the drug hits possess better physicochemical properties and higher safety. Even though, medicinal chemistry optimization are necessary to improve the antifungal activity and reduce the original therapeutic effects. Natural products are important source of antifungal lead compounds. Their complex structures and unfavorable physicochemical properties always limit further development. Identification and validation of new antifungal targets can greatly promote the drug discovery and development process. Thus, it is highly challenging to find a fungal-specific target and also a selective antifungal agent. Fortunately, important progress has been made in fungal genomes. Genome-scale analysis, comparative genomics and bioinformatics approaches have been used to find out potential targets unique to fungal cells [85,114]. Structural biology is also of great importance to determine the 3D structures of targets and thus facilitate the following structure-based drug discovery. However, crystal structures for most targets located in fungal cell wall or cell membrane still remain unknown. Currently, most of the new antifungal scaffolds were still discovered by traditional ‘compound-centric’ approach. In the post genomics-era, combining the ‘compound-centric’ and ‘target-centric’ strategies can accelerate the drug discovery process.

Future perspective

Although a number of screening hits with whole-cell antifungal activity have been reported, subsequent medicinal chemistry studies, such as SAR, lead optimization and mode of action, are still highly desirable. Moreover, these novel antifungal scaffolds can also be used chemical probes to identify antifungal targets by chemical genetics approaches. To fight against drug resistance, more efforts need to focus on new types of chemical scaffolds with fungicidal activity and new mode of action. The journey of a new chemical scaffold from conception to clinical application is a long one and it is estimated that most of the new antifungal agents marketed in the following years are still from existing classes (e.g., new triazoles and echinocandins). Even though, the application of new strategies, such as genomics-based target identification, new screening models, structural biology and rational drug design into antifungal drug discovery will accelerate the process of new antifungal drug development.

Financial & competing interests disclosure

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Executive summary

The urgent need to develop novel antifungal agents
• There is an emergent need to discover and develop new generation of antifungal agents with novel chemical scaffolds and fungal-specific mode of action due to high incidence and mortality of invasive fungal infections and lack of effective antifungal agents.

Discovery of novel antifungal scaffolds by high-throughput screening
• High-throughput screening is a major approach to discover novel antifungal scaffolds. The screening efficiency largely depends on the quality of compound library and antifungal assays.
• A number of in vitro (e.g., adenylate kinase-based and alamarBlue-based methods) and in vivo (e.g., Caenorhabditis elegans whole-animal model) assays have been developed to improve the quality of screening hits.
• Novel carboline, diazaspiro-decane, 1-benzoyl-4-phenylpiperazine and 4-(dimethylamino)styrylpyrylium scaffolds showed potent antifungal activity.

Discovery of novel antifungal scaffolds by drug repurposing
• A number of ‘nonantifungal’ drugs were identified to possess antifungal activity by screening drug libraries. Several of them, such as tamoxifen, amiodarone, pyrvinium, haloperidol and melengestrol, showed potent activity and need to be further optimized.

Discovery of novel antifungal scaffolds from natural products
• Natural products are featured as good structural diversity and are rich sources novel antifungal scaffolds. Due to structural complexity and unfavorable physicochemical properties, antifungal natural products need to be further optimized to improve potency and drug-likeness.
• Scaffold hopping and structural simplification of antifungal natural product sampangine led to the discovery of two novel scaffolds with facile chemical synthesis, potent antifungal activity and good water solubility.

Discovery of novel antifungal scaffolds based on new targets
• New inhibitors of glycosylphosphatidylinositol-anchored proteins, acetohydroxyacid synthase, dihydrofolate reductase and β-1,6-glucan synthesis showed potent antifungal activity.

Future perspective
• New strategies, such as genomics-based target identification, new screening models, structural biology and rational drug design, will be applied to antifungal drug discovery and accelerate the drug development process.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest
17 Balkovec JM, Hughes DL, Masurekar PS, Sable CA, Schwartz RE, Singh SB. Discovery and development of first


** A excellent review of antifungal drug resistance.


• Reports a new high-throughput screening assay of Candida albicans adhesion and discovered 1-benzoyl-4-phenylpiperezene scaffold with potent antifungal activity.


An excellent review of antifungal natural products.

A paper representing use of drug repurposing strategy to discover novel antifungal scaffolds.

A review on antifungal natural products.
Reports medicinal chemistry approach of the discovery of GPI anchor inhibitor E1210 as a new antifungal candidate.


• A good example of structure-based design of novel antifungal scaffold.


Fungal biofilm composition and opportunities in drug discovery

Biofilm infections are exceptionally recalcitrant to antimicrobial treatment or clearance by host immune responses. Within biofilms, microbes form adherent multicellular communities that are embedded in an extracellular matrix. Many prescribed antifungal drugs are not effective against biofilm infections owing to several protective factors including poor diffusion of drugs through biofilms as well as specific drug–matrix interactions. Despite the key roles that biofilms play in infections, there is little quantitative information about their composition and structural complexity because of the analytical challenge of studying these dense networks using traditional techniques. Within this review, recent work to elucidate fungal biofilm composition is discussed, with particular attention given to the challenges of annotation and quantification of matrix composition.

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Keywords: Aspergillus • biofilm • Candida • efflux pumps • fungal • immunoassay • NMR • persister cells • solid-state nuclear magnetic resonance

Aim

The majority of micro-organisms, including fungi, exist in nature as multicellular communities called biofilms [1]. Traditionally, most microbes have been studied as planktonic organisms. However, research into biofilm communities and biofilm composition and physiology is expanding given the recognition of biofilms as major contributors to microbial persistence and virulence in infectious diseases. Indeed, it is estimated that up to 80% of human microbial infections are biofilm-related [2]. Biofilms can be recalcitrant to antimicrobial treatment and can avoid clearance by host immune responses [1–5]. Both Candida albicans and Aspergillus fumigatus form biofilms, and these biofilms are involved in a range of human infections, including lethal fungal infections among immunosuppressed individuals [6–9]. C. albicans contributes to hospital-acquired infections and fouling of medical devices including catheters [5,6,10]. Biofilms formed by the opportunistic filamentous fungus A. fumigatus are also implicated in aspergilloma and invasive pulmonary aspergillosis (IPA) [11,12]. Additionally, both C. albicans and A. fumigatus have been found to co-infect with Pseudomonas aeruginosa in lung infections of patients with cystic fibrosis [13,14]. In a study of the prevalence of fungal species in the sputum of adult patients with cystic fibrosis, A. fumigatus was isolated from the sputum from 45.7% of patients in the study and C. albicans from 75.5% of patients [13]. Several additional fungal species are known to form biofilms and contribute to human infections [15–18].

A defining feature of a biofilm is the extracellular matrix (ECM), which is a self-produced, typically noncrystalline material that encases microbial cells [19,20]. The ECM is rich in biopolymers and can contain proteins, polysaccharides, lipids, nucleic acids, and other molecules [21] that can interact with one another and the cellular surface to form a robust, protective network [3,22,23]. ECM composition varies across species and even growth conditions [19,22], yet the ECM composition of many biofilms remains
unknown [19,21]. Functionally, the ECM can serve as a protective barrier against chemical and biological antimicrobial agents including many prescribed antifungal drugs (Figure 1A) [5,19–21]. In some instances, ECM can contribute to antifungal resistance by binding to antifungals, thereby preventing access to their intended target at the surface or within fungal cells [16,24–29]. As such, a better understanding of ECM composition is key to understanding the specific mechanisms of antifungal resistance exhibited by biofilm formers. Antifungal resistance has been studied extensively for C. albicans, and in some cases Candida biofilms have been found to be capable of withstanding antifungal concentrations that are 1000-fold higher than those that inhibit planktonic cells [5,30–32]. The presence of β-1,3-glucans in the ECM partially can explain this remarkable level of resistance as β-1,3-glucans can interact with a number of antimicrobials [33–37]. For example, radio-labeled fluconazole was found to be sequestered by ECM, which was correlated with the quantity of β-1,3-glucans in the matrix [30]. β-1,3-glucans can also interact with amphotericin B, flucytosine and echinocandins [30]. Interestingly, biofilm resistance to echinocandins is lower as compared with other antifungals, and it has been hypothesized that this may be because echinocandins impair β-1,3-glucan synthesis [30]. Indeed, mutants unable to produce the enzymes that transfer glucan from the cell to the extracellular milieu demonstrated reduced ECM glucan levels and decreased resistance to antifungal therapy [27]. Other ECM components, including extracellular DNA, also modulate drug resistance in biofilms produced by C. albicans and A. fumigatus, and in some instances, treatment of biofilms with DNase can reduce antifungal resistance [30,38–42]. A. fumigatus biofilms similarly exhibit decreased susceptibility to azoles, echinocandins and polyenes [43–45], including to amphotericin B. In addition, the co-administration of amphotericin B with alginate lyase, an enzyme that can degrade some exopolysaccharides within biofilms, was found to enhance the antifungal activity of amphotericin B against A. fumigatus biofilms, possibly by disrupting the polysaccharide component of the ECM and thus permitting drug access to hyphae [46]. These examples help to demonstrate the importance of having knowledge of ECM composition to provide insight into inhibitor modes of action and, ultimately, for developing strategies to combat the antifungal resistance exhibited by biofilms.

The ECM is often noted as a permeability barrier to antifungals that contributes to decreased drug susceptibility, although exceptions have been reported. One study utilized a disk diffusion assay to determine the C. albicans biofilm permeability of four antimicrobials: flucytosine, fluconazole, amphotericin B, and voriconazole [25]. Drug penetration through the biofilm failed to kill the cells, which suggested the existence of drug resistance mechanisms in addition to poor antifungal penetration for Candida biofilms. However, a previous study showed that the growth conditions (shaking above 30 rpm) used can inhibit ECM formation for C. albicans, as documented by scanning electron microscopy (SEM). Thus, further analysis is needed to probe effects on drug penetration [21,25,48]. Resistance can also be conferred to biofilm cells through the community’s harboring of persister cells (Figure 1B) [24,29,49–51]. Persister cells are metabolically dormant cells that exhibit tolerance to multiple antifungals including amphotericin B, azoles, and chlorhexidine [30,49,50]. Finally, biofilm microbes also upregulate the production of efflux pumps to confer enhanced antibiotic resistance relative to microbes not associated with biofilms (Figure 1C) [28,29,52]. Increased expression of efflux pumps during biofilm growth has been observed for several Candida spp. and A. fumigatus, and resistance to azoles, drugs that disrupt ergosterol synthesis, is frequently due to increased expression of efflux pumps [30,53,54].

The formidable survival traits of biofilms combined with our dwindling pipeline of effective antifungals make it imperative that we undertake in-depth analyses to improve upon our understanding of biofilms and ECM composition and function [55–57]. Indeed, standard antimicrobial susceptibility tests are still performed to examine the efficacy of single drugs using planktonically grown cultures, yet the biofilm state is often more relevant [3,24]. Early descriptions of biofilms were often limited to ‘slime’, and this simplistic description masked the complexity of biofilms and slowed the design of biofilm inhibitors and interventions [20,58]. The poor solubility and lack of crystallinity of biofilms and ECM render these materials especially challenging to examine using many traditional biochemical and biophysical techniques [19,20,59,60]. Despite these challenges, recent studies have developed the necessary tools to measure and define quantitative parameters of biofilms and the ECM [3,61,62]. Here we review two approaches to annotate ECM composition with specific attention given to C. albicans and A. fumigatus, the most well-studied fungal systems and common causative agents of fungal infections in hospitalized patients. The first approach implements an arsenal of traditional biochemical methods to identify important ECM components, including identification via immunostains [63–66]; and the second approach relies upon solid-state nuclear magnetic resonance (NMR) analysis of the intact ECM to profile the atomic-level contributions of unique classes of...
Fungal biofilm composition & opportunities in drug discovery

Review

Biomolecules to the ECM. Solid-state NMR uses the nuclear spin property to obtain information about chemical composition and structure, and can be used to examine any heterogeneous and insoluble materials, including whole cells and biofilm communities, and is not restricted to the study of soluble materials as many conventional methods are. The strengths and limitations of the two methods will be compared, and at the conclusion of this review, we discuss how these approaches might be used in the context of drug discovery to provide improved opportunities to effectively treat fungal infections.

Immunoassays to identify A. fumigatus ECM constituents

Specific biomolecules can be identified using a range of indicator dyes, antibodies, and lectins. Alternatively, the ECM constituents within each macro-molecular class can be annotated through systematic biochemical analysis without prerequisite knowledge of putative ECM composition. The first approach, using indicator dyes and antibody- and lectin-conjugated dyes, has been useful in fluorescence microscopy studies to identify and spatially resolve biofilm constituents, and recent advances in super-resolution microscopy have permitted the tracking of single proteins and polysaccharides during biofilm growth. These studies have taught us that many ECM constituents appear to play complementary architectural roles and that localization of components within a biofilm can vary over time. It is important to note, however, that an inherent limitation of these studies is that they require prior knowledge of the biofilm molecules of interest.

Immunoassays, which use antibodies that recognize specific molecules in order to identify or track the presence of that molecule, were useful to validate the presence of specific ECM polysaccharides and proteins in A. fumigatus biofilm, including biofilms formed in vitro and in vivo. Early work by Beauvais et al. revealed that the colony surface of A. fumigatus grown under aerial static conditions contained a hydrophobic ECM that bound hyphae in a manner that closely resembled in vivo ‘fungal balls’. Additionally, as predicted for biofilm, cultures grown under these conditions exhibited enhanced resistance to polyene antifungals relative to those grown under non-ECM-producing conditions (shaken, submerged). Interestingly, gentle mechanical removal of the ECM did not modify resistance to nystatin, which suggested that the A. fumigatus ECM may only reduce mycelial accessibility of some polyene antifungals, and in some cases additional biofilm-specific mechanisms may be responsible for antifungal resistance. Immunoassays and subsequent gas chroma-

Figure 1. A scanning electron micrograph of Aspergillus fumigatus biofilm reveals a network of extracellular matrix that surrounds agglutinated hyphae. Biofilms are recalcitrant to antifungal treatment, and several mechanisms have been proposed for this increased tolerance. (A) The extracellular matrix can serve as a physical barrier that blocks antifungals from accessing the fungal cells. An example is the binding of amphotericin B by β-1,3-glucans that is associated with decreased antifungal susceptibility. (B) Biofilms can harbor persister cells, which are metabolically dormant cells that exhibit increased antifungal tolerance. (C) Biofilms often have a relative upregulation of efflux pumps compared to their planktonic counterparts. The efflux pumps can expunge antifungals from the cells.

Adapted with permission from [47] © American Society for Microbiology (2015).
Glucose was identified as a major ECM component of *in vitro* *A. fumigatus* biofilms by monosaccharide analysis of digested ECM. Although the authors controlled for the possibility of contaminating glucose from the growth medium by culturing in glucose-free medium, it is important to recognize the general limitations of conditions used for monosaccharide analysis in many applications. To emphasize this, we highlight an example from work with the opportunistic human pathogen *Pseudomonas aeruginosa*. Until recently, Pel was determined to be a polymer of N-acetylgalactosamine and N-acetylgalactosamine [23]. This assignment was supported by the reactivity of Pel to antibodies raised against poly-β-1,6-N-acetylgalactosamine and chitosan (poly-β-1,4-N-acetylgalactosamine) as well as binding to N-acetylgalactosamine-specific lectins.

Protein quantification assays also have limitations in their application to biofilm and ECM samples. The overall protein concentration of *in vitro* *A. fumigatus* ECM was found to be 2% (w/w) by the bicinchoninic acid (BCA) assay. However, this assay can incorrectly estimate protein concentration, as the complex molecular environment of the ECM can contain biomolecules such as glucose and lipids that can cause interfering absorbance in the presence of the BCA reagent or may limit accessibility of the protein peptide bonds to the BCA reagent (specifically Cu^{2+} ions) [72-74]. The major antigens, DppV, catalase B and Asp f1, were identified by immunoblot, and the hydrophobic nature of the colony suggested the presence of hydrophobic proteins [75]. However, the contributions and identities of other potential proteins were not determined.

Despite the technical limitations of the above methods and inability to generate a complete accounting of the *A. fumigatus* ECM, the assays allowed an informative comparison between *A. fumigatus* ECM samples formed during host invasion, which was observed to depend upon whether the aspergillosis was localized (aspergilloma) or invasive (IPA) [64]. ECM was present in both pathological settings. However, hyphae in the IPA were separated and surrounded by a thinner layer of ECM relative to that of an aspergilloma. Aspergilloma appeared as a ball of strongly agglutinated hyphae, which was devoid of host cells and surrounded by a dense ECM network. Whole-biofilm immunoassays revealed that galactosaminogalactan (GAG) and galactomannan were major polysaccharides observed in the ECM, and α-1,3-glucan was only detected in aspergilloma at the periphery of the ECM. *In vivo* labeling with anti-GAG antibody was more intense as compared with *in vitro* labeling, suggesting that GAG was a major component of ECM produced during the development of *A. fumigatus* in tissues of patients with either aspergilloma or invasive aspergillosis. Later, it was observed that GAG mediates adherence to host cells, serves to control the host immune response by shielding β-glucans, and is a virulence factor that is required for biofilm formation [76-78]. The prevalence of GAG was later supported by studies using atomic force microscopy showing that GAG was highly exposed and able to serve as a fungal adhesin [79].

The biosynthesis of GAG has also been under study with efforts to understand the mechanism of deacetylation of residues in GAG, for example [80,81]. Immunochemical assays performed with antibodies raised against the three major secreted antigens mentioned above (DppV, catalase B, and Asp f1), showed that these antigens were not embedded in the *in vivo* ECM [64]. Thus, the immunoassays of putative polysaccharide and protein components permitted comparative analysis of the ECM from *in vitro* and *in vivo* *A. fumigatus* biofilms and demonstrated that each system included many similar components such as GAG, galactomannan, α-1,3-glucans, and melanin. In addition, some components, such as the antigenic proteins, were enriched in the *in vitro* ECM but not detected in the *in vivo* ECM [63,64]. These studies highlight the strengths of using immunoassays to detect particular predicted ECM components. However, this approach is limited to studying only predicted components as well as those for which specific antibodies are available. Furthermore, using this approach, it is not possible to quantify the contributions of each component in the total ECM.

**Macromolecular screening for *C. albicans* ECM composition**

Many of the biochemical methods available to identify ECM components were collectively implemented in a recent single study of *C. albicans* biofilm by Zarowski et al. (Figure 2) [65]. In this study, the researchers first profiled the contributions to the crude ECM of each of the macromolecular classes: protein, polysaccharide, lipid, and nucleic acid. This initial profiling was performed using a combination of spectrophotometric and colorimetric assays. Specifically, the protein enrichment was determined to be 55% (w/w) using a BCA assay; the carbohydrate content was determined to be 25% (w/w) using the phenol–sul-
furfuryl alcohol; the nucleic acid concentration was determined to be 5% (w/w) using the absorbance of the crude ECM at 260 nm; and, finally, the remaining 15% (w/w) was assigned to lipid. The crude ECM was then processed in order to further characterize specific C. albicans ECM constituents using additional biochemical techniques. Specific proteins were identified using proteomic mass spectrometry following enzymatic digestion of crude matrix material. Most of the 565 identified proteins play a role in metabolism or in the production and modification of polysaccharides. Interestingly, when the proteomic analysis was repeated using in vivo biofilms formed in a rat catheter model, the majority of identified ECM proteins were mammalian, suggesting that host factors may greatly impact biofilm composition, a topic that warrants further study. Sequence analysis found that much of the nucleic acid was noncoding. Lipids were profiled using gas chromatography, and nearly all of the lipids were found to be glycerolipids.

Further analysis of the carbohydrate contributions to the ECM primarily relied upon monosaccharide analysis and solution NMR following purification and fractionation steps. Initially, size-exclusion chromatography identified both high-molecular weight and low-molecular-weight fractions that constituted 38.3 and 61.7% of the total carbohydrate, respectively. Monosaccharide analysis concluded that the high-molecular weight fraction was primarily mannose, while the low-molecular-weight fraction was mostly arabinose. 1D and 2D solution NMR analysis of the abundant neutral carbohydrate fractions supported the presence of both mannans and glucans residues, and the most-abundant mannann polysaccharide was investigated using small-angle x-ray scattering. While 2D correlation NMR methods were not able to identify a linkage between the mannans and glucan residues, a novel mannann–glucan complex was predicted based upon the constant ratio of each in chromatography fractions as well as colocalization of anti-mannan and anti-glucan antibodies observed in confocal imaging of in vivo biofilms. This colocalization also was observed in biofilms formed in vivo. As previously mentioned, β-1,3-glucan can bind to antifungals. However, only small amounts of matrix β-1,3-glucan were observed via immuno-TEM, suggesting that additional ECM components are likely able to sequester antifungals. This concept was explored using a solution-state 1H NMR binding assay, in which line-broadening of matrix peaks suggested an interaction of the matrix with fluconazole. It was also determined that multiple ECM components were necessary for drug binding, which was further supported in a later study [82].

This meticulous study provided new information about the relative abundance of the different macromolecules in C. albicans ECM that could be digested and accessed, and used this information to predict the presence of a novel exopolysaccharide complex [65]. However, many biofilms are resistant to the solubilization that is required for such solution-based analyses, and harsh degradative conditions are often used to attempt to overcome this challenge and generate some material for analysis [83,84]. Furthermore, in addition to the previously stated limitations of the BCA assay to determine ECM protein concentration, the phenol–sulfuric acid method can incorrectly estimate the enrichment of carbohydrate. For the phenol–sulfuric acid method to be quantitative, the standard samples must contain representative portions of the types of monosaccharides found in the experimental sample, which is difficult to predict for complex carbohydrate mixtures such as ECM [85]. As a consequence of the limitations of these solution-based assays, estimates of contributions of proteins, polysaccharides, and other biomolecules can be dramatically misrepresented depending on the efficiency of the solubilization and the extent of material loss during sample processing, and extreme care should be taken when interpreting results from such analyses. These limitations, coupled with the importance of defining biofilm matrix composition as well as comparing biofilms and the influence of potential inhibitors, have encouraged the development of new approaches to help transform more qualitative biofilm descriptors into quantitative parameters of molecular composition [58,59].

**Solid-state NMR to quantify A. fumigatus ECM composition**

We recently developed an approach that utilizes solid-state NMR to quantitatively characterize and define biofilm and ECM composition [61,86]. Solid-state NMR, in general, permits analysis of the entire, intact ECM without preparatory chemical or enzymatic processing. There is no intrinsic size or mass limit as in solution NMR, which requires high-molecular tumbling rates to achieve high-resolution NMR spectra [61]. More specifically, and as reviewed in more detail in the direct context of biofilm and ECM characterization, solid-state NMR employs magic-angle spinning to mechanically spin samples of multicellular biofilm communities or isolated ECM to help achieve the necessary resolution to obtain quantitative spectra of these heterogeneous and insoluble systems [61,62]. Solid-state NMR has been applied to study other similarly complex and insoluble systems such as bacterial whole cells and cell walls [87–91] and intact plant leaves [92]. In 2013, we reported the first quantitative determination of the chemical composition of intact ECM of a microbial biofilm by using solid-
Figure 2. Schematic representation of a protocol employed to characterize the composition of *Candida albicans* biofilm. This methodology relies upon extensive processing of the biofilm and extracted matrix. Differences in solubilization efficiency of ECM components can result in compositional estimates that may vary widely from prep-to-prep [65]. An advantage of this approach, which uses an integrated effort, is the identification of specific matrix parts in the ECM, although there are caveats with quantification of these parts.

**C. albicans** biofilm

- **Matrix isolation**
- **Crude ECM**

**Purification and fractionation steps:**
- Size exclusion chromatography, anion exchange, and gel filtration

**Chemical analysis:**
- GC, NMR, and SAXS

- **Carbohydrate analysis**
  - Colorimetric determination
  - Approximate carbohydrate content

- **Protein analysis**
  - BCA assay
  - Approximate protein content

- **Lipid analysis**
  - Solvent extraction and methylation
  - Sterol determination
  - Fatty acid identification

- **Nucleic acid analysis**
  - Absorbance at 260 nm
  - Prostaglandin characterization

**Proteomic mass spectrometry (LC–MS/MS)**

**Monosugar identity and linkage determination**

**Protein characterization**

**Determined that eDNA is noncoding**

**Future Med. Chem.** (2016) 8(12) future science group

To overcome this limitation, we developed an alternative top-down solid-state NMR approach that permits spectroscopic dissection and annotation of complex material and can be applied to biofilms or other multicomponent systems for which there is less known (or even nothing known) about potential constituents [61]. We first developed this method to examine *Vibrio cholerae* ECM composition, and as discussed below, subsequently implemented the top-down approach in *Aspergillus fumigatus* [61,83]. This approach uses two types of 1D solid-state NMR experiments: cross-polarization magic-angle spinning (CPMAS) [93,94] and rotational-echo double-resonance.
(REDOR) [95]. The $^{13}$C CPMAS spectrum contains information about the quantities of carbon types, including carbonyls, aromatic carbons, polysaccharide carbons (anomeric and nonanomeric) and aliphatics, present in the ECM. By ensuring full incorporation of $^{15}$N into the matrix via defined growth medium, it is possible to further annotate the carbon pools according to C–N and C–P couplings using both $^{13}$C($^{15}$N) and $^{13}$C($^{31}$P)REDOR NMR experiments [61,83].

We applied this top-down solid-state NMR methodology to quantify the ECM composition of the fungal A. fumigatus biofilm grown in RPMI 1640 nutrient medium (selected because of its optimum for growing mammalian cells) [47]. The $^{13}$C CPMAS spectrum of A. fumigatus ECM showed contributions from a range of biomolecules (Figure 3A). To use CPMAS to quantify the number of nuclei at corresponding chemical shifts relative to others in a spectrum, differences in cross-polarization (CP) efficiency and relaxation must be taken into account. Such differences can arise due to variations in local dynamics and in the spatial distributions of $^1$H nuclei in the sample that are coupled to the carbons. By way of experimental detail, we monitored the A. fumigatus ECM $^{13}$C CPMAS intensities as a function of CP contact time, and observed a typical ‘exponential rise–exponential decay’ behavior that was extrapolated to zero contact time to quantify relative spin numbers. Together the $^{13}$C CPMAS peaks due to polysaccharide anomeric and ring-sugar carbons, which are chemical shift resolved and thus were able to be uniquely assigned, accounted for approximately 43% of the total carbon mass.

To annotate the remaining 57% of the carbon mass, we capitalized on the unique C–N and C–P couplings that are present in different biomolecules (Figure 3B & C). For example, the $^{13}$C spectrum of the ECM contained a peak near 173 ppm that was attributed to carbonyl carbons. Several types of biomolecules contain carbonyls, but all peptide carbonyl carbons are directly bonded to nitrogen (Figure 3C). Thus, by using $^{13}$C($^{15}$N)REDOR as a spectroscopic filter to select only carbonyls that are directly bonded to nitrogen, we could determine an upper limit of the amount of protein carbonyls. Through determining the quantitative CPMAS peak intensities of different carbon types as described above, we discovered that the carbonyl peak accounted for 12% of the total $^{13}$C spectrum of A. fumigatus ECM. Using $^{13}$C($^{15}$N)REDOR, we found that only 76% of the carbonyl carbons were directly bonded to nitrogen. Thus, at most, only 9% (76 of 12%) of total ECM carbon could be attributed to peptide carbonyls of proteins. The percentage of possible α-carbons was similarly determined to be 7% (including α-carbons of both glycine and other amino acids). Amino acids contain an average of 5.4 carbons each, and so three additional carbons can be generally attributed to protein. These carbons mostly would be observed in the upfield aliphatic region of the $^{13}$C CPMAS spectrum. Together, this analysis supports that approximately 40% of the total carbon mass can be attributed to protein. The aromatic region of the $^{13}$C CPMAS spectrum accounts for an additional 3% of the total carbon mass, which was partially attributed to melanin.

The remaining 14% of the $^{13}$C CPMAS spectral area contains contributions from carbonyls and aliphatics (excluding directly nitrogen-bonded carbons), including a sharp peak at 33 ppm that is characteristic of CH$_2$ groups typically found in lipids and accounts for 7% of the total spectral area. Spectral selection of portions of these regions by $^{13}$C($^{31}$P)REDOR also suggested that the ECM contained some phospholipid. Thus, A. fumigatus ECM contained at least 7% lipid (due to the characteristic peak at 33 ppm) and up to 14% lipid by carbon mass. Taken together, the A. fumigatus ECM produced under these growth conditions was approximately 40% protein, 43% polysaccharide, 3% aromatic-containing components and up to 14% lipid.

Thus, atomic-level parameters of the intact isolated A. fumigatus ECM were measured and defined using the top-down solid-state NMR method. In addition, other valuable compositional parameters of the ECM constituents were obtained. The $^{15}$N CPMAS spectrum showed that most of the ECM nitrogen is present in protein (appearing as an amide peak at 119 ppm). We were also able to detect low abundance nitrogen-containing motifs likely present as modifications to ECM constituents, for example, as amino or N-acetyl modifications. The $^{13}$C($^{15}$N)REDOR results suggested that some of the exopolysaccharides were N-acetylated based upon the presence of peaks attributed to N-acetyl methyl groups and the observed proximity of the nitrogen to some of the sugar-ring carbons. This finding is consistent with some of the previously identified extracellular polysaccharides produced by A. fumigatus including GAG. N-acetyl groups also could occur as part of the N-acetylglycosamine groups present in N-linked glycosylation of ECM proteins. The possibility that some of the extracellular proteins were glycosylated was explored using traditional biochemical analysis, SDS-PAGE analysis of the ECM showed several bands corresponding to SDS-soluble proteins, and these bands were excised and subjected to proteomic mass spectrometry. The SDS-soluble proteins were identified as catalase B and Asp f2. Consistent with the NMR spectral detection of N-acetyl groups, these two proteins are reported to be N-linked glycosylated. Digestion with peptide-N-glycosidase F (PNGase) resulted in cleavage consistent with glycosylation of Asp f2 in this ECM sample.
Figure 3. *Aspergillus fumigatus* extracellular matrix composition determined using solid-state nuclear magnetic resonance. (A) The $^{13}$C cross-polarization magic-angle spinning spectrum of *A. fumigatus* extracellular matrix showed carbon contributions from a range of biomolecules including proteins, carbohydrates and lipids. (B) The contributions of specific carbon pools to the total carbon mass were annotated using cross-polarization magic-angle spinning and spectral editing via $^{13}$C($^{15}$N) and $^{13}$C($^{31}$P)REDOR nuclear magnetic resonance experiments, and these contributions are summarized in the graph. (C) Chemical structures of representative biomolecules that were identified in the *A. fumigatus* extracellular matrix. Adapted with permission from [47] © American Society for Microbiology (2015).

pared across samples, whether due to different biofilm formers or the influence of external stimuli such as antibiofilm agents. We developed an approach to finely annotate the specific carbon types and to obtain a quantitative accounting of these pools in the biofilm ECM. Even quick inspection of the comparative 1D CPMAS spectra would permit facile identification of whether any significant differences were present among a sample set. Coupled with electron microscopy of intact biofilms and the isolated ECM material as well as biochemical analyses for the specific identification of some biofilm parts, we believe that this integrated approach with solid-state NMR analyses provides one of the most robust and powerful ECM characterization approaches available.

**Conclusion**

Connections between ECM composition and function are crucial to understanding the fundamental molecular basis for: fungal biofilm physiology, the recalcitrance of biofilm infections to antifungal treatment, and antifungal resistance. Robust and reliable methods to define and/or quantitatively compare ECM composition are crucial for driving these connections. The two major approaches to annotate ECM composition described in this review provide complementary details of ECM composition and connections with function. Solid-state NMR is uniquely suited to profile chemical composition of complex, insoluble systems like ECM [62]. The top-down solid-state NMR method described above can be applied to the study of any biofilm, including mixed species or *in vivo*-derived biofilms, and, importantly, does not require any prerequisite knowledge of biofilm composition [47,61,62,83].

The approach first involves the annotation of the fundamental carbon and nitrogen compositional pools, for example, quantifying the prevalence of carbonyls and methyls, etc. It also takes advantage of potentially unique internuclear couplings to further dissect...
 ECM composition and to specify the types of carbonyls present, for example. The analysis is performed on the intact ECM and provides parameters not accessible from solution-based biochemical methods that require solubilization of ECM parts, wherein much of the ECM is often not analyzed due to its recalcitrance to digestion and dissolution. Furthermore, solid-state NMR analysis permits one to simultaneously observe and quantify multiple macromolecular species and a complete analysis can be performed on the same sample. The advantages of the solid-state NMR approach thus include the nondestructive nature of the analysis and the ability to quantify the types of nuclei present in a sample. A disadvantage, is the limited sensitivity of NMR and the relatively large sample sizes and/or long spectral acquisition times required to achieve high signal-to-noise spectra using natural abundance $^{13}$C samples. Isotopically labeled samples improve sensitivity, although it is attractive that $^{13}$C profiling, for example, does not require labeling and can be performed using $^{13}$C at natural abundance. NMR correlation experiments provide additional selectivity to specify the specific nature of carbon pools that are annotated through 1D spectra. These experiments require isotopic enrichment, for example, as we demonstrated with $^{19}$F-incorporation in a customized RPMI 1640 medium for the analysis of A. fumigatus ECM composition described above [47]. Thus, it is important to choose the right tool for the experiment, taking into account the problem-solving advantages and the technical limitations that can include sample solubility, processing requirements, and sample size.

**Future perspective**

Ideally, the traditional biochemical methods applied to solubilized parts of the ECM should be integrated with quantitative compositional profiling by solid-state NMR. The NMR analysis provides a total snapshot of molecular contributions to the intact ECM and the prevalence of molecules in the ECM, such as the relative abundance of proteins versus polysaccharides, including an analysis of types of chemical modifications present in the samples. Yet, while each protein has a unique NMR signature, identification of individual proteins in the ECM is best performed using proteomic mass spectrometry and immunoassays. Similarly, the identities of lipids and small, soluble polysaccharides can be determined using solution-based biochemical methods on the individual purified components in isolation from one another. In addition to the study of complex, biomolecular mixtures, solid-state NMR can be applied to study in detail the isolated ECM components such as the high-molecular-weight polysaccharide fractions identified in the previously described C. albicans study [65], and does not require harsh, degradative conditions prior to analysis. In this way, solid-state NMR has been used to quantitatively determine structural information of similarly noncrystalline and insoluble bacterial [88,90,91,96] and plant cell walls [97–100].

Solid-state NMR also is well suited for the study of drug–matrix interactions, with inspiration from studies that mapped atomic-level interactions of the antibiotic $[^{19}$F]oritavancin with S. aureus cell walls (both in isolated cell walls and in the whole-cell context) by measuring several $^{19}$F-$^{13}$C and $^{19}$F-$^{15}$N distances between drug and specific cell-wall sites [87]. In the realm of antifungals, solid-state NMR approaches have been used to examine the possible mechanism(s) of action of amphotericin B in lipid vesicle systems used as surrogates for fungal membranes. Most structural studies have characterized the pore-forming assemblies of amphotericin [101], yet very recent solid-state NMR work examined samples prepared with phospholipids, ergosterol and amphotericin, and showed that amphotericin B could extract ergosterol out of phospholipids, and serve as a type of ergosterol sponge [102]. These types of structure-focused NMR approaches are ripe for identifying specific ECM components that bind to antifungals and for mapping the interactions between the ECM and $^{19}$F-labeled drugs, for example. Together, solid-state NMR methods enable the quantification of the abundance of biomolecules in a biofilm and can be used to determine the ways in which the components could be interacting with antifungals to contribute to the antifungal resistance often exhibited by biofilms.

The ECM is able to inhibit the access of some antifungals to fungal cells in a biofilm and contributes to the challenge of treating biofilm-associated infections. The potential mechanisms of ECM-mediated antifungal recalcitrance include both matrix–matrix interactions and matrix–drug interactions. For example, the matrix could be self-associating to form an impermeable barrier to drugs. Alternatively, the matrix could either bind to drugs or enzymatically inactivate the drugs, both of which prevent antifungals from reaching their intended target at the surface of or within the fungal cell. The ECM composition plays a key role in dictating the possible mechanisms that biofilms employ to decrease susceptibility to antifungals. In the past, knowledge of ECM composition has given rise to more effective strategies to treat biofilm-involved fungal infections such as the coadministration of amphotericin B with alginate lyase to degrade exopolysaccharides and enhance the antifungal activity of amphotericin B against A. fumigatus biofilms [46]. As knowledge of biofilm composition, including composition of mixed...
Executive summary

Background
• Biofilm infections are recalcitrant to antimicrobial treatment and clearance by host immune responses.
• Many prescribed antifungal drugs are not effective against biofilm infections owing to several protective factors including poor diffusion of drugs through biofilms as well as specific drug–matrix interactions.
• Both Candida albicans and Aspergillus fumigatus form biofilms, and these biofilms are involved in a range of human infections, including lethal fungal infections among immunosuppressed individuals.
• A defining feature of biofilms is the extracellular matrix (ECM), which is a self-produced, noncrystalline material that encases microbial cells. ECM is typically rich in biopolymers and can contain proteins, polysaccharides, lipids, nucleic acids, and other molecules.
• The formidable survival traits of biofilms combined with our dwindling pipeline of effective antifungals make it imperative that we undertake in-depth analyses to improve upon our understanding of biofilms.
• The two approaches, traditional biochemical methods and a newly developed solid-state NMR method, can be used to annotate ECM composition and provide complementary perspectives on ECM composition and function.

Immunoassays to identify A. fumigatus ECM constituents
• Immunoassays were useful to validate the presence of specific ECM polysaccharides and proteins in A. fumigatus biofilm, including both in vitro and in vivo biofilms.
• Immunoassays and subsequent gas chromatography identified galactomannan and α-1,3-glucans as A. fumigatus ECM components.
• The major antigens, DppV, catalase B and Asp f1, were identified by immunoblot in the in vitro ECM but were not detected in the in vivo ECM.

Macromolecular screening for C. albicans ECM composition
• The ECM of Candida albicans was examined and the contributions of each of the macromolecular classes were profiled.
• This initial profiling was performed using a combination of spectrophotometric and colorimetric assays, and the ECM was determined to be 55% (w/w) protein, 25% (w/w) carbohydrate, 5% (w/w) nucleic acid, and 15% (w/w) lipid.
• Carbohydrate characterization primarily relied upon monosaccharide analysis and solution nuclear magnetic resonance (NMR) following purification and fractionation steps, and both high-molecular-weight and low-molecular-weight fractions were identified.
• Many biofilms are resistant to the solubilization that is required for such solution-based analyses, and harsh degradative conditions are often used to overcome this challenge which can lead to misrepresentations of ECM composition.

Solid-state NMR to quantify A. fumigatus ECM composition
• A solid-state NMR approach was developed that permits analysis of the entire, intact ECM without preparatory chemical or enzymatic processing.
• Solid-state NMR does not require high tumbling rates in solution (solution NMR) or homogeneous samples, and provides quantitative information about composition.
• This solid-state NMR approach uses two types of 1D NMR experiments: cross-polarization magic-angle spinning and rotational-echo double-resonance.
• Spectral dissection using solid-state NMR determined that the A. fumigatus ECM was approximately 40% protein, 43% polysaccharide, 3% aromatic-containing components, and up to 14% lipid.
• In addition to the atomic-level parameters of A. fumigatus ECM gained using this top-down solid-state NMR method, general characteristics of the ECM constituents were obtained including glycosylation of extracellular proteins and types of modifications of exopolysaccharides.

Opportunities in drug discovery
• Measurements of ECM composition are crucial to understanding fungal biofilm physiology, the recalcitrance of biofilm infections to antifungal treatment, and antifungal resistance.
• Integrated approaches employing electron microscopy, biochemical methods including proteomics analyses, together with solid-state NMR compositional measurements would allow for comprehensive characterization and elucidation of the generation of quantitative parameters of ECM composition, enabling comparisons with samples from organisms treated with antifungal and antibiofilm agents.
• Solid-state NMR approaches can be used to map the ECM binding sites and bound conformations of candidate therapeutics in isolated ECM and in intact cells.
species and in vivo biofilms increases, we believe that effective strategies to treat biofilm-involved fungal infections will also improve. Knowledge of the ECM composition provides evidence of which mechanisms could be in play for a particular biofilm, and furthermore, provides routes to overcome the matrix-mediated antifungal resistance. These routes may involve combined antifungal and anti-virulence approaches. For example, the biophysical and biochemical ways in which the ECM is able to act as a drug barrier could be taken into account when designing an antifungal drug so that the drug meets size, charge, and reactivity requirements to be able to pass through the biofilm and impact the fungal cells. Complementary anti-virulence approaches could block matrix–matrix or matrix–drug interactions, either through co-administration of additional drugs or through mechanical disruption. Specific knowledge of those matrix–matrix and matrix–drug interactions is crucial to the design of such therapeutics. Thus, compositional and molecular-level descriptions of the ECM should help to drive the development of strategies to eradicate biofilm-associated infections and develop more effective antifungal treatments.

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**References**

Papers of special note have been highlighted as:

* • of interest


• Review of fungal biofilm composition.


23 Jennings LK, Storek KM, Ledvina HE et al. Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the
Review Reichhardt, Stevens & Cegelski


• Review of antimicrobial resistance associated with biofilms.


• Key paper that outlines the ‘top-down’ solid-state nuclear magnetic resonance (NMR) method to study extracellular matrix (ECM) composition. Describes the analysis of A. fumigatus ECM.


50 Rajendran R, Mowat E, McCulloch E et al. Azole resistance of Aspergillus fumigatus biofilms is partly associated with...


• Review that compares two solid-state NMR methods for analysis of biofilm composition.


• Review that details the use of the ‘bottom-up’ solid-state NMR method to quantify ECM composition.


• Key paper to describe *A. fumigatus* ECM composition.


• Key paper to describe *in vivo* *A. fumigatus* ECM composition.


• Key paper that outlines many of the biochemical methods available to study ECM composition. Describes the analysis of *C. albicans* ECM.


84 Yildiz F, Fong J, Sadowskaya I, Grasd T, Vinogradov E. Structural characterization of the extracellular polysaccharide


