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Antifungal therapy with an emphasis on biofilms

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Abstract

Fungal infections are on the rise as advances in modern medicine prolong the lives of severely ill patients. Fungi are eukaryotic organisms and there are a limited number of targets for antifungal drug development; as a result the antifungal arsenal is exceedingly limited. Azoles, polyenes and echinocandins, constitute the mainstay of antifungal therapy for patients with life-threatening mycoses. One of the main factors complicating antifungal therapy is the formation of fungal biofilms, microbial communities displaying resistance to most antifungal agents. A better understanding of fungal biofilms provides for new opportunities for the development of urgently needed novel antifungal agents and strategies.

INTRODUCTION

Fungal infections are increasing at an alarming rate affecting an expanding population of severely ill patients which poses important challenges for the practising physician [1]. *Candida* spp., *Aspergillus* spp. and *Cryptococcus neoformans* are among the most common etiologic agents of fungal infections, but infections caused by other yeasts and moulds are on the rise. Unfortunately, the mortality rates associated with these mycoses remain unacceptably high, very often higher than 50% [1,2], which clearly indicates major shortcomings and limitations in the clinical management of patients with fungal infections. Moreover, these devastating infections place an additional financial burden on our already strained health care system [3,4]. Despite these facts the field of antifungal drug development has mostly stagnated in the last decade, as major pharmaceutical companies have focused their efforts in more profitable drugs to treat chronic conditions. This article aims to illustrate current antifungal therapy and its shortcomings, particularly with the recent recognition that a majority of fungal infections are associated with biofilm formation [5], which in turn results in increased resistance to most conventional antifungal agents and complicates treatment. We will also discuss recent developments in the field of fungal biofilm research, which may offer new hope for the development of novel antifungal drugs.

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ANTIFUNGAL AGENTS

Fungi are eukaryotic organisms evolutionary close to humans and, as such, there are a limited number of targets which can be exploited for antifungal drug development. As a consequence, the current antifungal armamentarium is still very limited, and the antifungal pipeline is essentially dry [6,7]. This paucity of “selective” targets is also the main reason for the elevated toxicity frequently associated with antifungal therapy. Three classes of antifungal agents, namely azoles, polyenes and echinocandins, constitute the mainstay of antifungal therapy for patients with life-threatening invasive mycoses [6](Figure 1A). Although these antifungal agents have demonstrated their efficacy by improving patient health in human medicine, they also have significant shortcomings.

Since their discovery and introduction in the 1950s polyenes, and particularly Amphotericin B, remained the “gold standard” of antifungal therapy for decades. These amphipathic compounds bind ergosterol, the principal sterol in the fungal membrane, and create pores that compromise membrane integrity causing leakage of cellular contents and death (Figure 1A). However, the efficacy of polyenes is severely limited by their intrinsic toxicity, particularly nephrotoxicity, but also infusion-related toxicity. Several lipid formulations of Amphotericin B have been subsequently developed to try to minimize these toxicity concerns [6,7].

Azoles, first developed in the 1980s, are the largest class of antifungal agents used today in clinical medicine. They include some topical agents but also systemic triazoles. Azoles are fungistatic drugs that inhibit ergosterol biosynthesis: they target 14 α -lanosterol demethylase (Erg11 or Cyp51), a cytochrome P-450 enzyme which catalyzes a key step in the ergosterol biosynthetic pathway [6,7](Figure 1A). In contrast to fluconazole, whose activity is mostly limited to yeasts, itraconazole, voriconazole and posaconazole display good activity against filamentous fungi (*i.e. Aspergillus*). The improved safety profile compared to Amphotericin B impelled the use of azoles for a variety of therapeutic indications. However a major problem with azoles has been the emergence of resistance (including cross-resistance against multiple azole derivatives) [8–10]. Important mechanisms leading to azole drug resistance are the development of point mutations on the target enzyme and the overexpression of genes encoding efflux pumps, both major facilitators and ABC transporters [11]. These studies have also unraveled a high degree of regulation, mostly at the transcriptional level, of drug resistance genes in fungal pathogens [12].

The new millennium brought a brand new class of antifungals to the market, the echinocandins (*i.e.* caspofungin, micafungin and anidulafungin) [6,7]. Echinocandins are actually the first class of antifungal drugs that act against a specific component of the fungal organisms – the cell wall - not present in mammalian (host) cells (Figure 1A). These are a group of semisynthetic lipopeptide antibiotics whose mechanism of action is the specific and non-competitive inhibition of the 1,3- β -D-glucan synthase complex. This enzyme is critical for the synthesis of the key structural glucan polymers of the fungal cell wall, and its inhibition leads to depletion of cell wall glucan and lysis of fungal cells [13]. Echinocandins display excellent fungicidal activity against most *Candida* spp. They are considered fungistatic against *Aspergillus* spp. and some other moulds, for which voriconazole and amphotericin B are generally preferred therapeutic options. However, the spectrum of action of echinocandins does not include *Cryptococcus* or other emerging pathogens. Although echinocandins represent a welcome addition to the antifungal arsenal due to their excellent safety profile, emergence of resistance, mostly due to mutation in the *FKS1* gene encoding the target enzyme, glucan synthase, has been observed despite their relatively recent introduction into the clinics [8,13,14].

FUNGAL BIOFILMS AND ANTIFUNGAL DRUG RESISTANCE

One of the main factors further complicating antifungal therapy in an increasing number of patients is the ability of fungal cells to form biofilms [5,15]. A biofilm is defined as a structured microbial community attached to a surface and encased within a self-produced extracellular matrix [16]. Today it is well established that biofilms are the predominant form of microbial growth in nature, including during infection [17–19]. Among pathogenic fungi, *Candida* spp. are the most frequently associated with biofilm formation [19,20]. Different types of biomaterials often used in the clinics support colonization and biofilm formation by *Candida*, most notably intravascular catheters [19,21,22]. Recent studies indicate that *Aspergillus* spp. also have the ability to produce multicellular structures which point to a role for biofilms in different manifestations of these infections (including endocarditis associated with prosthetic valves) [23–25]. *Cryptococcus neoformans* can colonize and subsequent form biofilms on ventricular shunts, peritoneal dialysis fistulas, and cardiac valves [5]. Other yeasts and filamentous fungi whose biofilm-forming ability associated with clinical settings has been described include *Coccidioides*, *Pneumocystis*, *Malassezia*, *Penicillium*, *Histoplasma*, *Saccharomyces*, *Trichosporon*, *Blastoschizomyces*, and some zygomycetes [5,15,26]. Contrary to *C. albicans*, which is only found inside its host, some of these fungi are ubiquitous in nature, and it is conceivable that biofilm formation can also contribute to their survival under hostile environmental conditions and against predation [27,28]. Although traditional methods for the formation of fungal biofilms were cumbersome, most recently research on fungal biofilms has been greatly facilitated by the development of relatively simple methodologies; most notably the 96-well microtiter plate model for the formation of *Candida* biofilms [29,30], which was subsequently adapted for *Cryptococcus*, *Aspergillus* and other fungi [24,28]. These methodology can also be easily adapted for antifungal susceptibility testing of fungal biofilms (see below) [29]. Also, different groups of investigators have developed a variety of animal models, particularly in the case of *Candida* biofilms [31–36]. From a structural point of view, in general mature fungal biofilms exhibit a complex three-dimensional structure and extensive spatial heterogeneity, with a typical microcolony/water channel architecture, and are encased within exopolymeric material [37,38]. Fungal biofilm development occurs through different phases, including initial attachment, proliferation, maturation and ultimately dispersion so that the “biofilm life-cycle” can be repeated all over again [37–39]. In the case of *C. albicans*, biofilm formation is inextricably linked to morphogenetic conversions and adhesive interactions (both for the attachment to the substrate and for intercellular interactions), with extracellular matrix accumulation as the biofilm matures, and the whole process is finely controlled by quorum sensing mechanisms and, at the molecular level, by a complex regulatory network [40–48].

From a clinical perspective fungal biofilm formation carries important negative consequences, as they provide a safe sanctuary for microorganisms, act as reservoirs for persistent sources of infections, as well as potentially adversely affecting the function of implanted devices [5,19,22]. Cells within these biofilms display properties that are dramatically different from planktonic (free-living) populations, most notably high-level resistance to most antifungal agents [49]. From the very early reports it was demonstrated that fungal biofilms show intrinsic resistance to azole derivatives, as well as displaying high levels of resistance against polyenes (both Amphotericin B and Nystatin) [30,50]. In reality, polyenes display good biofilm activity but unfortunately they do so at concentrations which are high and generally considered toxic and unsafe [51]. Subsequent reports indicated excellent activity of liposomal formulations of Amphotericin B against *C. albicans* biofilms [52]. Very importantly, echinocandins seem to display excellent anti-biofilm activity at therapeutic concentrations [51–53], at least in the case of *C. albicans*; although other fungal biofilms, including those formed by *A. fumigatus* biofilms, are relatively resistant to

echinocandins [15,49]. The antifungal drug resistance exhibited by sessile fungal cells within biofilms is multifactorial (Figure 1B) and different contributory mechanisms include: i) the increased cellular density within the biofilm (safety in numbers); ii) the existence of subpopulations of “persister” cells; iii) the metabolic and physiological status of cells; iv) the distinct sterol composition of the cell membrane of sessile cells; v) the protective effect of the biofilm matrix, including drug binding by exopolymeric components (*i.e.* glucans binding azoles); vi) cellular stress responses mostly mediated by Hsp90; and vii) the differential expression of genes linked to resistance, particularly those encoding efflux pumps (for an excellent review including the detailed description of mechanisms of resistance please refer to [49]).

NOVEL APPROACHES TO TARGET FUNGAL BIOFILMS

As discussed above, there is a clear urgent and unmet need to develop novel strategies, for the prevention and treatment of infections associated with the formation of fungal biofilms, and certainly this is an area of very active research. A possibility is to combine a conventional antifungal agent with another drug that potentiates its antibiofilm activity. For example, both Hsp90 and calcineurin inhibitors may be able to overcome resistance when used in combination with antifungals [54,55], and DNase treatment improves the anti-biofilm activity of conventional antifungal agents (as the fungal biofilm matrix contains extracellular DNA) [56]. Also, a high throughput screening technique was used to identify potentiators of the clotrimazole biofilm activity [57]. There is also increased interest in the examination of the anti-biofilm activity of natural products [58]. Repurposing already existing drugs can represent a viable and faster alternative for the identification of drugs with novel antifungal activity; for example we screened the Prestwick library consisting of 1,200 FDA-approved off-patent drugs and identified several inhibitors of *C. albicans* biofilm development [59]. In the particular case of catheter-associated infections, a possibility is to use catheter lock solutions (suprapharmacological concentrations of drugs locally inside the catheter lumen) [60], and also to develop biomaterials and coatings which do not support fungal biofilm growth [61]. Other potential strategies include, among others, the use of modulators of quorum sensing, antimicrobial peptides, and photodynamic therapy [62–64].

CONCLUSION

Fungal infections constitute an escalating problem negatively impacting the wellbeing of an ever expanding number of immunocompromised patients that ultimately translates to a soaring financial burden to our health care system. The high mortality rates associated with these infections clearly indicates that current antifungal therapy is less than ideal: the limited arsenal of antifungal drugs together with toxicity problems and emergence of resistance pose immense challenges to clinical practitioners. Most of these infections are associated with biofilm formation, which further complicates treatment since cells within biofilms showed increased levels of resistance against most antifungal agents. The increased recognition and attention by the medical and research communities to the role played by these microbial communities during infection should lead to a better understanding of fungal biofilms and may offer new opportunities for the development of novel antifungal agents with activity against biofilms and to overcome fungal biofilm resistance. These new strategies will offer new hope for patients suffering from these devastating infections.

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HIGHLIGHTS

- Fungal infections affect an expanding population of immunosuppressed patients.
- The current arsenal of antifungal drugs is exceedingly limited.
- Toxicity problems and resistance limit the efficacy of current antifungals.
- Formation of fungal biofilms further complicates antifungal treatment.
- There is an urgent need to devise strategies to overcome biofilm drug resistance.

A. Antifungal agents and their targets

B. Multifactorial nature of biofilm antifungal drug resistance

Figure 1.

A. An schematic diagram illustrating the mechanisms of action, at the cellular level, of the most important classes of clinically-used antifungal agents. Polyenes bind to ergosterol in the fungal cell membrane; azoles block ergosterol biosynthesis; and echinocandins inhibit the synthesis of β -glucan thereby affecting the fungal cell wall. **B.** A scanning electron micrograph of a fungal biofilm and the multiple factors contributing to biofilm antifungal drug resistance.