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Overcoming antifungal resistance

Anand Srinivasan^{1,3}, Jose L. Lopez-Ribot^{2,3,*}, and Anand K. Ramasubramanian^{1,3,*}

¹Department of Biomedical Engineering, The University of Texas at San Antonio, San Antonio, TX78249

²Department of Biology, The University of Texas at San Antonio, San Antonio, TX78249

³Department of South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, TX78249

Abstract

Fungal infections have become one of the major causes of morbidity and mortality in immunocompromised patients. Despite increased awareness and improved treatment strategies, the frequent development of resistance to the antifungal drugs used in clinical settings contributes to the increasing toll of mycoses. Although a natural phenomenon, antifungal drug resistance can compromise advances in the development of effective diagnostic techniques and novel antifungals. In this review, we will discuss the advent of cellular-microarrays, microfluidics, genomics, proteomics and other state-of-the art technologies in conquering antifungal drug resistance.

Keywords

Fungal infections; antifungal drug resistance; resistance mechanisms; biofilms; Fungal infections; microarray; biofilms; high-throughput; drug screening; antifungals

INTRODUCTION

The origin of fungi can be dated back to more than 1,500 million years [1]. Although highly diversified, only 0.5 % of fungal species are considered to be pathogenic to humans [2]. This population of pathogens includes the causative agents of aspergillosis, candidiasis, coccidioidomycosis, cryptococcosis, histoplasmosis, mycetomas, mucormycosis, and paracoccidioidomycosis, among others. Until the 19th century most infectious diseases were attributed to bacterial, parasitic and viral origins and perhaps any less-frequent cases of

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*Address correspondence to: Anand K. Ramasubramanian, Ph.D. Department of Biomedical Engineering The University of Texas at San Antonio San Antonio, TX 78249 Phone: 210-458-6555 Fax: 210-458-7007 anand.ramasubramanian@utsa.edu Jose L. Lopez-Ribot, Pharm.D./Ph.D. Department of Biology The University of Texas at San Antonio San Antonio, TX 78249 Phone: 210-458-7022 Fax: 210-458-7023 jose.lopezribot@utsa.edu.

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fungal diseases that could have prevailed before the 1800s were not recognized or documented [3]. After the discovery of antibiotics, clinicians observed unusual microbial colonization and disease symptoms during antibiotic therapy. Slowly the possibility of fungi to cause disease in humans became apparent.

It was reported that the severity of infections caused by fungal organisms could range from moderate to fatal, also depending on the site of infection and the immune status of the patient. Moderate fungal infections including cutaneous infections such as ring worm and athlete's foot are common in many individuals, including immunocompetent patients. On the other hand, the mucosal and systemic infections are often "opportunistic"; that tend to manifest when the immunity is compromised, and often lead to life-threatening infections. Some of the most common causative agents of these opportunistic mycoses are *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. The common targets of these pathogens have been immunocompromised individuals such as patients suffering from HIV-AIDS, diabetes, cystic fibrosis and cancer, among others [4,5].

Although targeting a small group of patients compared to viral or bacterial counterparts, these infections are often associated with high morbidity and mortality rates [6]. Based on a study conducted by National Institute of Health, in the United States alone between 1980 – 1997, the mortality rate due to invasive mycoses has increased by 320% [7]. The study also reveals that the incidence of fungal infections has paralleled with the increase in the number of immunosuppressed individuals, organ transplants, autoimmune disorders and carcinoma. The reason for high mortality and morbidity are poor diagnosis, emergence of drug-resistance and lack of effective antifungal therapy. Worsening disease severity and further complicating treatment, some fungi can form "biofilms" that display elevated resistance to most conventional antifungals [8]. Similar to bacteria, biofilms are the major cause of intractable fungal infections in immunocompromised patients with indwelling catheters, heart valves, pacemakers and other biomedical-assist devices. This review will focus on the current antifungal drugs in practice and the emerging drug resistance that contribute to persistent infections with a particular emphasis on technologies that could be employed to overcome antifungal drug resistance.

Antifungal drugs

The development of antifungal therapeutics poses a sizable challenge as fungi are eukaryotes like the human hosts they infect. This leaves few distinct targets that can be employed for antifungal drug development. In the late 1950s intravenous formulations of polyenes such as Amphotericin B and Nystatin B were developed as the first generation of antifungals. Polyenes target ergosterol; a component that is analogous to cholesterol in mammalian cell membranes. Nevertheless, these compounds were compromised with high toxicity in humans [9]. Two decades later, a major breakthrough was made with the development of "azoles" as intravenous and oral formulations. The azoles target the biosynthetic pathway of ergosterol by inhibiting an early-phase enzyme called lanosterol 14 α -demethylase encoded by ERG11 [10]. This leads to the accumulation of sterol intermediates that pose toxic stress to the fungal cell; making the cells vulnerable to membrane damage. Nevertheless, the fungistatic nature of the drug combined with their

ability to interact with the Cytochrome P 450 enzymes made them less amenable for therapy in patients undergoing multidrug therapy. These limitations paved the way for the development of “echinocandins” in 2001. These drugs are semi-synthetic derivatives of a cyclic lipophilic peptide isolated from *Glarea lozoyensis*. Echinocandins are fungicidal drugs that block cell-wall synthesis by inhibiting β -(1, 3)-D-glucan synthase [11].

Despite the accessibility of these antifungals there is a steady increase in the morbidity and mortality associated with invasive mycoses. This prompted physicians and microbiologists to investigate on the phenotypic properties, including resistance and cell fitness, of isolates causing recurrent infections in AIDS patients. These studies led to the identification of drug-resistant strains against azoles in immunosuppressed HIV-infected patients, as well as organ transplants, catheters, implants and other biomedical device associated infections. In addition to that, the indwelling devices harbored a community of microorganisms encapsulated in an exopolymeric matrix called biofilms. Cells disseminated from these biofilms due to dispersion served as a source for invasive mycoses [Srinivasan, 2013 #55; [12]. A fully formed biofilm is recalcitrant to antifungal therapy and often demands surgical intervention.

Antifungal resistance mechanisms

Despite the availability of antifungal agents; the incidence of mycoses, tolling death rate and complexity of treatment can mostly be attributed to the emergence of resistance to antifungal therapy. This condition is exacerbated especially in patients with compromised immunity. For instance, in the US as of November 2013 with 19,262 organs transplants there was a concomitant increase in the susceptibility of these immunocompromised patients to invasive fungal infections [13,14]. In order to develop better treatment strategies it is important to understand the antifungal drug-resistance mechanisms developed by fungal cells. Antifungal resistance is observed when the growth of the pathogen is unaffected at a therapeutic concentration of the antifungal agent. The phenomenon of drug resistance can be due to multifaceted reasons such as increased efflux of the drug, phenotypic alterations in the drug target site, genomic recombinations that minimize toxic effect of the drug and biofilm formation. Since each class of antifungals act via different mechanisms, they need to be discussed separately.

As shown in Table 1, there are multiple ways in which fungi could develop drug resistance and azoles are notorious for their high predisposition to lead to resistance through multiple mechanisms, most notably overexpression of efflux pumps and changes in the target enzyme [15]. Although drug-resistance towards polyenes is relatively rare, several studies have reported that non-*albicans* species of *Candida* such as *glabrata*, *krusei*. and particularly *lusitaniae* are intrinsically resistance or have a higher propensity to develop polyene-resistance [16,17]. Other invasive fungi exhibiting intrinsic resistance to amphotericin-B are *Aspergillus terreus*, *Trichosporon beigellii*, *Fusarium* sp., *Scopulariopsis* sp. and dematiaceous moulds, with mortality rates higher than 70% [18-20]. Echinocandins are often effective for the treatment of fungal infections caused by azole-resistant isolates. Nevertheless, the increasing emergence of strains resistant to echinocandins can be

considered as a clear sign for the urgent need to search for the next-generation of antifungals [21].

Apart from these drugs, allylamines, antimetabolites and pyrimidine analogs have also been used since 1970s. Nevertheless, the fungal strains rapidly developed resistance by mutation and interference from cellular drug transporters [22]. For this reason, clinicians resort to combination therapy with azoles or amphotericin B. Administration of antifungal combinations is very rare because of high risk factors such as multi-drug interactions, toxicity and some concerns over potential antagonistic effects. Hence clinicians bank on it as recourse for only severe cases of mycoses.

As mentioned before, biofilm formation by fungi leads to high levels of resistance against most clinically used antifungal agents. In this case, the mechanisms of resistance are drastically different as compared to those displayed under planktonic conditions discussed above. In general, the antifungal drug resistance exhibited by cells within the biofilms is multifactorial, with multiple contributory mechanisms that may include the presence of the biofilm matrix, the metabolic and physiological status of cells, the presence of “persisters”, the increased number of cells within the biofilms, differential gene expression of genes linked to resistance (i.e. efflux pumps), different sterol composition, and stress responses. For more detailed information, readers are referred to two excellent recent reviews on this topic, by Ramage *et al* and Mathe *et al* [23,24].

Overcoming antifungal resistance

The evolution of antimicrobial drug resistance is an almost inevitable process that is ubiquitous in the microbial world. Although the development of antifungal drug resistance has not paralleled their antibacterial antibiotic counterparts, the economic facets associated with fungal infections remains unacceptably high. In addition to this, the arsenal of antifungal agents is extremely limited; therefore overcoming antifungal resistance can be considered as the mainstay for improving therapeutic strategies to treat mycoses [25]. One of the major factors exacerbating antifungal-drug resistance is the inappropriate use of antifungals, forcing the fungi to evolve against selective pressure. Numerous studies have elucidated the association of improper antifungal drug exposure and emergence of resistance. Thus antifungal therapy should employ effective agents to alleviate promotion of drug-resistance. Furthermore, the dose and spectrum of action of the antimicrobial agent has important ramifications because of their potential effects on the human microbiome. It has been identified that the imbalance in the host microbial diversity imparts deleterious effect and sometimes development of tolerance in less-susceptible fungi. Considering these factors, the potential strategies to embark in order to overcome antifungal resistance will be discussed further.

Early diagnosis

One of the major reasons for high mortality and morbidity for mycoses could be attributed to the delay in diagnosis and institution of appropriate therapy [26]. Diagnosis of fungal pathogen deep-seated in the tissues or organs is almost impossible and is often driven by clinical suspicion. Unless systemic, any detection of fungemia is only apparent due to

dissemination of cells from the invaded tissues, organs or biofilms; at which point it is already overdue. Current practices involve histopathology analyses and phenotypic examination of the colonies cultured using selective agar plates. Histology analyses of biopsy samples are untenable as they lack specificity, sensitivity and taxonomic information. In other cases fluid samples such as blood, alveolar lavage, and sputum or tissue counterparts are first cultured on non-selective medium to maximize microbial counts for 24 hours and later typed for selection using genus-specific agar plates. In spite of the delay to acquire results, the assay can only provide limited qualitative information. However, in patients suffering from life-threatening fungal infections, institution of appropriate (type and dose) antifungal therapy becomes of critical importance for the outcome and also to hinder the development of antifungal drug-resistance.

From a clinical perspective, the choice of diagnostic technique is rather complicated. For instance, immunocompromised patients suffering from thrombocytopenia, neutropenia or pulmonary aspergillosis cannot often be subjected to biopsy or other conventional diagnosis [27]. In such cases biomedical imaging techniques like computed tomography, x-ray radiography and magnetic resonance imaging are used as confirmatory tests [28]. Another example includes studies that have reported biopsy-positive fungemic patients with no fungal burden in their blood samples [29]. In such circumstances a clinician has to choose between time-consuming confirmatory tests that may delay therapy versus broad spectrum antibiotic, risking antifungal drug-resistance. A similar conundrum arises when a clinician deals with non-sterile samples which pose difficulty in differentiating colonization from contamination. Such scenarios demand a deep knowledge of the clinician in recognizing disease signs and symptoms [30].

Owing to such uncertainties, clinical samples are often subjected to *in vitro* antifungal susceptibility testing as a part of the diagnosis [31]. Such concurring techniques are time consuming and “low-throughput” since they are limited by simultaneous detection of multiple modalities. On the bright side, early diagnosis and concomitant therapy has proven to significantly improve patient outcome [32]. Evidently, these shortcomings in the diagnosis of fungal infections highlight the need for early and effective diagnostic techniques with increased specificity and sensitivity. Some of the impending surrogate diagnosis techniques target specific components rather than the whole cell. These non-culture-based techniques detect cellular components such as mannan and galactomannan (both antigen and antibody detection), D-arabinitol, nucleic acids and β -glucan. Although promising, these assays are still in an investigational phase requiring further validation in order to demonstrate increased specificity, sensitivity, positive predictive value and standardized protocols for approval from FDA [33-37].

The search for novel antifungals

The development of novel antifungals is challenging because fungi are eukaryotic organisms like mammals. With the exception of the cell wall, fungal cells share many similarities with the host thereby leading to a paucity of fungal-specific targets that can be exploited for antifungal drug development. Hence antifungals are mostly restricted to four drug classes targeting three metabolic pathways. The rationale of designing a drug based on the

biochemical structure of the target is time consuming when there is an urgent need. A rather empirical approach followed by some researchers and industrial laboratories involves screening of synthetic and semi-synthetic chemical libraries, natural extracts from plants, marine life and already existing medical compounds (repurposing) for antifungal properties. After assessing their effect on fungal viability, the interaction of the drug with its cellular target is investigated. Traditionally these assays were performed using broth or agar-based tests where the fungus was cultured in the presence of antifungal agent discs or mixed in the broth. The susceptibility of the fungal pathogen to the action of the drug was often measured and interpreted using appropriate break points as per a broth macro-dilution technique adopted by the Clinical Laboratory Standards Institute (CLSI). Despite being simple and convenient, these assay methodologies operated in milliliter scale of volume and lacked throughput in screening vast chemical libraries. During the 1980s and 1990s, these screens were replaced with well plate-based assays in which the fungus was cultured in micro-liter scale niches called “wells” (often referred as “broth micro-dilution technique, also standardized by CLSI). The cellular response to the antifungal agent was calculated by means of growth or, alternatively, a colorimetric read-out, in particular when measuring susceptibility of fungal biofilms [38]. A few years later, the wells were miniaturized to operate in few tens of microliters as observed in 384 or 1536 well plates. Such platforms were later handled using automated stations to minimize labor and reduce assay time. Nonetheless, the assays were cumbersome in handling *in vitro* susceptibility testing, including under both planktonic and biofilm growing conditions.

Addressing these issues, our group developed a “next-generation platform” for *in vitro* ultra-high-throughput drug screening (Fig. 1). The microarray consists of a standard microscope glass slide containing up to 1,200 individual *C. albicans* biofilms (‘nano-biofilms’), each with a volume of approximately 30 nanoliters, encapsulated in a three dimensional hydrogel matrix to simulate an environment which is close to the natural physiology [39]. Despite a 3,000-fold miniaturization over conventional biofilms formed on microtiter plates, these “nano-biofilms” are similar in their morphological, architectural, growth and phenotypic characteristics, including aspects of antifungal drug resistance. This platform is robotically-controlled and operates in nano-scale volume that combines to cut reagent cost, assay duration, labor and analysis time by simultaneously screening thousands of compounds in a single screen [40,41]. Such techniques can accelerate the screening for novel antifungal and anti-biofilm drugs of other species; thereby potentiating rapid, effective and economical drug screening.

Besides identifying novel candidates with antifungal properties, some of these techniques have also intended to identify compounds that potentiate the activity of azoles. For instance, selective inhibitors of calcineurin, efflux pumps and heat-shock protein 90 have been identified this way [42,43]. Another screen has used a collection of mutant strains to tease the effect of the drug in a particular mutation [44]. Such assays identified genes related to the mechanism of drug action.

In contrast to addressing drug resistance at the cellular or molecular level, a recent study by Pukkila-Worley *et al* uses whole-organism approach. In this model *Caenorhabditis elegans* infected with *C. albicans* were tested against a drug library in a high-throughput fashion

[45]. This model has advantages of identifying effect of candidates on fungal viability, virulence, toxicity and host-elicited immune response. An alternative approach targets elements responsible for pathogenesis; namely virulence factors that aid in the progression of infection. Drug candidates identified and developed using this anti-virulence will by definition display a narrow spectrum drug, but should also exert a much weaker selective-pressure thereby minimizing the potential for the development of drug-resistance [46].

Predicting the development of drug-resistance

So far we discussed about the means and measures to overcome antifungal resistance but, can we predict the emergence of drug-resistance? We must acknowledge that just like evolution, the development of resistance against a drug is almost inevitable. Nevertheless, the process of drug discovery would be less difficult if we could predict the propensity of a cells or organisms to develop drug-resistance, measure the timeline of emergence as well the factors the mediate such development. One such study conducted by Barlow-Hall predicts the evolutionary potential of a gene to develop resistance [47]. In this technique the authors generated all possible sequence variants of a gene target using artificial chromosomal recombination. The pool of variants were tested for drug-resistance using *S. cerevisiae* as a fungal vehicle. The idea was to use an organism with known molecular and genomic information that would facilitate the impact of these variants in the presence of an antifungal agent. The technique provided a better understanding of the plausible drug targets in biological networks. Although the study was aimed at predicting antibiotic resistance, similar efforts could theoretically predict emergence of antifungal drug-resistance before clinical manifestations.

Conclusion

It is apparent that the escalating trend of mycoses, emerging drug-resistance and poor patient outcome are unmet by the antifungals used in clinical practice. The ability of fungi to develop resistance against a drug is an evolutionary process and cannot be abrogated. Nevertheless, effective measures of managing antifungal resistance could be derived from a comprehensive understanding of drug-resistance mechanisms. Such efforts include, but are not limited to, delaying the development of antifungal resistance by advancing better diagnostics for early detection of infection, preemptive treatment, prophylaxis, combination drug therapy and identifying new antifungals. With the advent of ultra-high-throughput screening techniques, the hunt for novel and perhaps better antifungals should be possible. Future investigations could focus on identifying novel class of drugs directed against targets that do not impart selective pressure or promote drug-resistance. Identifying such targets should be feasible from genomic studies assisted with computational models.

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Figure 1. A microarray of nano-scale biofilms for ultra-high-throughput drug screening
 (a) A microarray scanner image of *C. albicans* nano-biofilms stained using FUN-1. A section of the nano-biofilm in xy, xz and yz planes with spatial measurements. (b) A picture depicting the side view of the biofilm microarray with the projection of a confocal micrograph of the nano biofilm.

Table 1

Antifungals and most frequent drug resistance mechanisms.

Antifungals	Mode of action	Mechanism of action	Mechanism of resistance	Ref.
Polyenes Amphotericin B Nystatin	Fungicidal	Binding to sterols in cell membrane forming aqueous pores	Reduction of ergosterol concentration ablating drug-target binding Alteration in POL gene family	[16,17,48,49]
Azoles Fluconazole Itraconazole Ketoconazole Posaconazole Voriconazole Miconazole	Fungistatic	Affects ergosterol biosynthesis by blocking the key enzyme, lanosterol 14 α -demethylase (Erg11p)	Efflux of drug by multi-drug transporters; ABC gene family Amino acid substitution to Erg11p affecting drug-target binding Over-expression of Erg11p minimizing effect of drug Change in toxic-sterol concentration due to mutation in Erg3 alleles	[25,50-53]
Echinocandins Caspofungin Micafungin Anidulafungin	Fungistatic or Fungicidal	Binding to β -(1, 3)-D-glucan synthase and inhibition of β -(1, 3)-D-glucan, a cell-wall component.	Mutation in Fks1 and Fks2 binding units	[54]
Allylamines Terbinafine Naftifine	Fungistatic	Interferes with ergosterol biosynthesis by inhibiting squalene epoxidase (Erg1p)	Interference from multidrug transporters	[55]
Pyrimidine analogs 5-Flucytosine	Fungicidal	Inhibits cellular DNA and RNA synthesis affecting molecular machinery	Mutation in cytosine permease and deaminase	[22]