Drug resistance in fungi — an emerging problem

Arunaloke Chakrabarti*

Abstract
Over the past quarter of a century, invasive fungal infections have emerged as an important cause of morbidity and mortality in immunocompromised patients. Although several new antifungal drugs have been licensed in recent years, antifungal drug resistance is becoming a major concern during treatment of such patients. The resistance may be intrinsic, acquired or clinical. The understanding of the mechanism of resistance and clinical impact is important while planning treatment strategies. Four altered gene expression pathways have been identified in azole resistance. The mechanism of resistance in polyene and echinocandins is still not clearly understood. Recent studies have revealed that molecular chaperone heat shock protein (Hsp90) can alter the relationship between genotype and phenotype leading to a profound impact on antifungal drug resistance. Though definite progress has been made to correlate standardized in vitro antifungal susceptibility testing with prediction of treatment outcome, limitations still exist due to time required for testing and understanding the factors leading to clinical resistance. Overall, the level of resistance to antifungal agents is still relatively low, but there is a possibility of antifungal resistance becoming a crucial determinant of outcome following antifungal therapy in future.

Introduction
Medical progress has led to an expanding population of susceptible hosts with impaired immunological defenses against infection in the community and hospitals. These populations are at heightened risk for many opportunistic fungal diseases including candidiasis, aspergillosis, mucormycosis (zygomycosis), cryptococcosis, pneumocystosis. Traditionally Candida and Aspergillus species accounted for the majority of infections. Candidemia is the fourth leading cause of blood-stream infections and carries 35-55% mortality¹. The incidence of mould infections has also increased in the recent past, especially infectious caused by Aspergillus spp. where the mortality rate crosses 50% in such patients². Mucormycosis is a threat in uncontrolled diabetes in developing countries like India³. In this scenario, contemporary epidemiological trends also indicate a certain shift of the fungal pathogen towards resistant species among those common two genera, Candida and Aspergillus, and emergence of the previously uncommon fungi that are particularly difficult to manage⁴,⁵. These include C. glabrata and C. krusei in yeast with their reduced drug susceptibility, and among the mould fungi, these include the non-fumigatus Aspergillus spp. like Aspergillus terreus, zygomycetes and Fusarium spp. Selective pressure due to increased use of antifungal prophylaxis in high-risk patients has been suggested as a contributory factor for this shift and emergence of uncommon mould⁶.

Antifungal drugs and the problem of resistance
For a long time amphotericin B deoxycholate and 5 fluorocytosine were the only therapeutic options for invasive fungal infections. The first
therapeutic alternatives began to emerge with the introduction of fluconazole and itraconazole in the late 1980s. Expansion in antifungal research in the last two decades has led to the development of lipid formulations of amphotericin B (amphotericin B colloidal dispersion, amphotericin B lipid complex, and liposomal amphotericin B), a second-generation broad spectrum triazoles (voriconazole, posaconazole) and an entirely new class of antifungal agents, the echinocandins (caspofungin, anidulafungin and micafungin). A few of the new antifungal agents are under clinical trial (Table 1). Despite the increase in the spectrum of antifungal agents now available, the choice of suitable antifungal agents remains relatively limited due to the emergence of comparatively more resistant fungal species, slow mycological diagnosis, variable drug bioavailability in immunocompromised patients, toxicity of antifungal agents, lack of either oral or intravenous preparations, drug interaction, and most importantly due to development of resistance and breakthrough infections7, 8.

Unfortunately, the increased use of triazoles in prophylactic and empiric antifungal therapy in high-risk patients has led to selective pressure towards drug-resistant Candida and Aspergillus species9. It has resulted in infection either through the inherently resistant fungi (primary resistance) or through the resistant subpopulation of the normally susceptible fungi (secondary resistance). Fortunately, development of acquired resistance in fungi is not a “fast-track” event as in bacteria or viruses, except in the event of nearly one third patients with advanced AIDS harbouring fluconazole-resistant C. albicans in their oral cavity9. As no known mechanism of horizontal resistance gene transfer was known in fungi, it was believed that exceptionally large number of viable fungi when exposed to high levels of antifungals in the oropharyngeal candidiasis might become resistant to antifungal agents10. The episode of rapid emergence of antifungal resistance ended with the advent of effective antiretroviral therapy in patients with AIDS. However, there is no scope for complacency as recently in a genome-wide analysis of three Fusarium species, it was shown experimentally that complete chromosomes could be transferred between different fungal strains12. Prior to this it was believed that fungi were generally confined to vertical gene transfer, a slower type of genetic change based on mutation, recombination and the effect of

<table>
<thead>
<tr>
<th>Compound</th>
<th>Currently available</th>
<th>Under clinical trial</th>
<th>Target site</th>
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<tbody>
<tr>
<td>Polyenes</td>
<td>Amphotericin B deoxycholate and lipid formulations (amphotericin B lipid complex, amphotericin B colloidal dispersion, liposomal amphotericin B)</td>
<td>Liposomal nystatin</td>
<td>Ergosterol in cell membrane</td>
</tr>
<tr>
<td>Fluorinated pyrimidine</td>
<td>5 fluorocytosine</td>
<td></td>
<td>DNA, RNA synthesis</td>
</tr>
<tr>
<td>Triazoles</td>
<td>Fluconazole, itraconazole, voriconazole, posaconazole</td>
<td>Isavuconazole, ravuconazole, albvoranazole</td>
<td>Ergosterol biosynthesis - 14α demethylase</td>
</tr>
<tr>
<td>Echinocandins</td>
<td>Caspofungin, anidulafungin, micafungin</td>
<td>Aminocandins</td>
<td>1-3-β-d glucan synthesis in cell wall</td>
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<tr>
<td>Allylamines</td>
<td>Terbinafine</td>
<td></td>
<td>Ergosterol biosynthesis – squalene epoxidase</td>
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selection. This new understanding of fungal genetics would help researchers understand the types of fungi that are most likely to develop resistance to antifungal agents.

The overall resistance in Candida spp. to fluconazole and voriconazole is considered to be around 3-6% and level of resistance has remained constant over a decade. However, a recent report from India revealed panazole resistance in ~10% of Candida species. Triazole resistance in A. fumigatus is increasingly being recognized and up to 6% of clinical isolates were found to be resistant to triazole in the United Kingdom and the Netherlands. In contrast to azoles, echinocandin resistance does not seem to be the major cause of concern, as global surveillance studies indicate that there has not been any significant epidemiological shift in the susceptibility of Candida spp. isolates to echinocandins. However, since 2005 there have been multiple case reports of breakthrough infections after echinocandin therapy in patients with AIDS or acute myeloid leukaemia. The prevalence of flucytosine resistance in yeast remains low (<2%). But the speed at which yeast can develop resistance to flucytosine has prompted clinicians to use the compound in combination with mainly amphotericin B. Overall, though the incidence of antifungal resistance is low, it remains a serious problem in the management of high-risk patients. Recently, concern has been expressed on the possibility of induction of resistance in opportunistic fungi in the environment as azole fungicides are used in agriculture.

### Mechanism of antifungal resistance

The mechanism of drug resistance in microorganisms traditionally takes the path of either identifying a cellular determinant that prevents entry of the drug or removes the drug from the cell or inactivates the drug or prevents the drug from inhibiting the target of various combinations of the above-mentioned pathways. In fungi, mutation in gene encoding target proteins, up-regulations of expression of multidrug efflux pumps and drug target themselves, altering the stoichiometry of the inhibitor target ratio in favour of fungus are possible mechanisms. However, no fungus has yet been shown to have the ability to degrade an antifungal agent like beta-lactamase in bacteria. Multidrug resistance, called pleiotrophic drug resistance (POR) in Saccharomyces cerevisiae is possibly an ancient model for multidrug resistance that operates in pathogenic fungi through the efflux pump. Therefore, most studies on the antifungal drug resistance mechanism have targeted the efflux pump mechanism. Inhibition of the pump over-expression or drug pump activity may transform a fungistatic drug like azole into a fungicidal drug. In C. albicans a unique mechanism of gene amplification leading to azole resistance has been identified. The mechanism involves formation of aneuploidy or isochromosome, in which the chromosome arm bearing both transcription factor (regulating ABC transporter) and target of the azoles Erg11 is duplicated. The different genetic alterations and mechanism of resistance in Candida spp. and Aspergillus spp. are summarized in Table 2.

#### Table 2: Genetic mechanism of resistance in Candida and Aspergillus (modified from reference 9)

<table>
<thead>
<tr>
<th>Antifungal Azoles</th>
<th>Candida</th>
<th>Aspergillus</th>
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<tbody>
<tr>
<td>- Decreased drug concentration (efflux pumps)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ CDR gene of ATP binding cassette (for all azoles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑ MDR gene of major facilitator class (for fluconazole)</td>
<td>Mdr1, Mdr3, Mdr4</td>
<td></td>
</tr>
<tr>
<td>C. albicans[CDR1, CDR2, MDR1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. glabrata[CDR1, PDH1, Snq2]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. dubliniensis[CDR1, CdMDR1]</td>
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### Antifungal Azoles

**Candida**

- Target cell alteration
  - Mutation of ERG11
  - Decrease affinity ERG11p (intrinsic resistance to fluconazole in *C. krusei* isolates)
  - ↑ ERG11p

**Aspergillus**

- Cyp51A (mutation at codon 220 develops resistance to all azoles, mutation at codon 54 develops cross-resistance to itraconazole and fluconazole)
  - ↑ Cyp51A

- Bypass pathway
  - Mutation of ERG3 (prevent formation of toxic products from 14-α methyl fectosterol)

**C. albicans** – chromosome 5

**Polyenes**

- Target site alteration
  - Mutation of ERG3 (accumulation of other sterols)
  - Alteration of drug : target ration by any mechanism

**Echinocandins**

- Target site alteration
  - Point mutation mostly at Ser 645 of Fks1
    - *C. albicans* Fks1
    - *C. glabrata* Fks1, Fks2
  - Activation of salvage or compensatory pathway for chitin synthesis (PKC cell integrity pathway)

From the evolutionary perspective, none of the mechanisms acts alone. Phenotypic resistance depends on the genetic variation occurring in a particular genome. However, the development of resistance is often accompanied by a deleterious effect of mutation on the fitness of fungi in the absence of the drug. Compensatory mutation may mitigate this effect and enhance fitness. Hsp90 is known to play an important role in remodelling the relationship between phenotype and genotype in distant species. In antifungal drug resistance its role has been emphasized recently. Hsp 90 acts as a capacitor for accumulation of genetic variation. When its function is compromised by genetic alteration, pharmacological inhibitors or environmental stress, genetic variations are revealed, which lead to alteration of the relationship between genotype and phenotype. Hsp90 acts through calcineurin. Any inhibitor of Hsp90 or calcineurin would possibly act synergistically with the antifungal agent.

### Antifungal drug susceptibility testing

Both the Clinical Laboratory Standard Institute (CLSI), United States of America, and the
European Committee on Antimicrobial Susceptibility Testing (EUCAST) have published approved protocol antifungal susceptibility testing either by broth microdilution or disc diffusion assay. Drug threshold levels for in vitro growth inhibition yield a minimum inhibitory concentration (MIC). The CLSI has recommended antifungal MIC breakpoints to separate susceptible and resistant population for azoles and echinocandins by analysing the in vitro susceptibility data, in vitro outcome and pharmacokinetics/pharmacodynamic studies. However, EUCAST defined the breakpoint derived from MIC as the Epidemiological Cut-off Value (ECV) to avoid confusion with clinical breakpoints. The EUCAST uses ECV “as the most sensitive measure of resistance development — for measuring resistance development in hospitals and the community, for measuring the effect of interventions and for developing strategies to counteract further resistance development”. The breakpoint derived through MIC tends to be lower than the clinical breakpoint, as this procedure is independent of dosage regimens. In contrast, the clinical breakpoint is based on distribution of MIC, pharmacokinetics of the antimicrobial agent, and the clinical outcome. Therefore, “the clinical breakpoint should be used in every day clinical laboratory work to provide evidence for rational therapy in the patient”. While correlating the therapeutic outcome in multiple studies with in vitro antifungal susceptibility testing data, especially the combination of Candida species and azole antifungal agents, a pattern of “90-60” rule emerged like in bacteria: infections due to susceptible isolates respond to therapy —90% of the time, whereas infections due to resistant isolate respond —60% of the time. However, in spite of all these studies and recommended standards, antifungal susceptibility testing rarely influences management protocol in an individual patient, as it takes 48-72 hours after isolation of the fungus. Therefore, there is a need for a more rapid test procedure or “real time” antifungal susceptibility testing for clinicians.

Cross-resistance among antifungal agents

Cross-resistance among azoles is expected as the target of action on fungi is similar. In HIV-positive patients a high level of cross-resistance to itraconazole was observed in fluconazole–resistant C. glabrata and C. tropicalis compared with C. albicans and C. krusei isolates. Cross-resistance in C. glabrata strains was due to increased expression of CgCDR1, CgCDR2 genes and CDR efflux pumps. Though voriconazole also has cross-resistance with other azoles, the rate is low, and after performing in vitro susceptibility testing it may be used in patients who have previously been exposed to fluconazole or itraconazole. However, in a study conducted in India, high cross-resistance to fluconazole, itraconazole and voriconazole was reported in C. albicans and C. tropicalis blood isolates, though the mechanism of resistance was not studied. Cross-resistance has been observed among the three echinocandins. Cross-resistance should not be expected between the echinocandin class of drugs and either the polyene or azoles, as the sites of action are different.

Clinical antifungal resistance

Non-specific symptoms and signs of invasive fungal infections present difficulties in early diagnosis; delay in diagnosis is the major cause of treatment failure. Even in empiric and targeted therapy the success rate ranged from 32% to 74%. The major causes of treatment failure have been summarized by Kanafani and Perfect: (i) incorrect diagnosis of specific fungal disease including immune reconstitution inflammatory syndrome (IRIS) in patients with AIDS after antiretroviral therapy; (ii) failure of antifungal agents to overcome the state of severe immune deficiency in such patients; (iii) more virulent infection such as Cryptococcus gattii infections; (iv) toxicities of antifungal agents (nephrotoxicity in polyene and hepatitis in azoles; (v) poor penetration of antifungal agents at certain sites of fungal
infections such as the central nervous system or the necrotic tissue with poor blood supply; (vi) reduced blood concentration of the antifungal agent due to drug interaction, especially during voriconazole therapy; (vii) suboptimal duration of the antifungal therapy; and (viii) the underlying disease as the main barometer of clinical success and failure in antifungal therapy.

Like bacteria, fungi also produce a biofilm in vitro. It is well known that the biofilm is an important obstruction in antibacterial therapy. In fungal infections similar studies have been conducted. Enhanced extracellular matrix especially beta glucan synthesis during biofilm growth has been shown to prevent penetration of antifungal agents such as azole and polyene. It is believed that the echinocandins and lipid formulations of amphotericin B can penetrate biofilm better than amphotericin B deoxycholate and azoles. The clinical trials also indicate the importance of the biofilm. Numerous clinical trials on candidemia have demonstrated that the treatment failure and mortality are high in patients who are on catheters for long periods.

Conclusion

With increase in the incidence and spectrum of invasive fungal infections, antifungal drug resistance has become an important consideration in the management of patients. Though unlike bacteria, the level of resistance to antifungal agents is relatively low due to the possible absence of drug-resistant plasmid or transposons in fungi, the recently conducted experiment of horizontal gene transfer in pathogenic Fusarium species shows that there is no scope of complacency. The emergence of intrinsically resistant fungal species as a human pathogen is compounding the challenge of planning treatment strategies. Beyond these confounding factors, the conditions leading to clinical resistance should be kept in mind while managing invasive fungal infections in immunocompromised patients.

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References and bibliography


