Review

Antifungal drug resistance: does it matter?

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The objectives of this review are: to review the modes of action of currently available antifungal drugs; to define drug resistance and discuss the mechanisms by which fungi can develop resistance to antifungal drugs; to consider the epidemiological and host factors that contribute to the outcome of antifungal therapy and whether the available in vitro susceptibility test methods can reliably predict clinical response; and to assess the overall relevance of drug resistance to the outcome of fungal infections.The incidence of antifungal drug resistance among pathogens causing invasive fungal infections appears to be increasing. In the case of *Candida* spp., this may in part be a consequence of selective pressure brought about by more intensive antifungal use leading to a 'pathogen shift'. Non-*albicans Candida* spp. are more likely to demonstrate reduced susceptibility to fluconazole compared to *C. albicans*. Susceptibility breakpoints developed by the National Committee for Clinical Laboratory Standards to test azoles and flucytosine against *Candida* spp. are helpful in guiding therapy. Antifungal drug resistance in yeasts is of clinical importance. Increasingly reliable methods of in vitro susceptibility testing can help predict clinical response to therapy, but other considerations, including host- and drug-related factors, can also have an important bearing on the ultimate outcome of treatment.

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INTRODUCTION

Antifungal drugs are widely prescribed both in the community and in hospital practice. Despite recent developments, there is still a limited repertoire of licensed antifungal agents, especially for the treatment of invasive fungal infections (IFIs). This is in the face of clear evidence of an increase in the incidence of IFIs in susceptible patient groups. These include the classical immunocompromised populations such as bone marrow transplant (BMT) and organ transplant recipients, human immunodeficiency virus (HIV)-positive patients and, increasingly, patients who are critically ill on high-dependency units. The specific indications for antifungal drugs in these groups are becoming better delineated, and current recommendations are supported in many cases by comparative clinical trial data.[1–3] For example, in patients with hematological malignancy, antifungal drugs are commonly administered for prophylaxis during neutropenia, empirical therapy of febrile episodes unresponsive to antibiotics, therapy of documented infections, and prevention of relapse during further immunosuppression where there is a history of previous fungal infection. Over the past two decades, antifungals have been widely used in HIV-positive patients for treatment of oropharyngeal /oesophageal candidiasis (OPC) and cryptococcosis. Patients on intensive care units are viewed as being at increased risk of invasive candidiasis, so that in this setting a role for systemic antifungal therapy is rapidly becoming established. It is not surprising, then, that the increased use of systemic antifungals has had an impact on the epidemiology of fungal infections that are frequently seen, and this has coincided with the emergence of drug resistance, especially in patient groups where the level of use is more intensive. Up until the past 5 years, there was no widely accepted method for in vitro susceptibility testing of fungal pathogens. With the publication by the National Committee for Clinical Laboratory Standards (NCCLS) in 1997[4] of a validated method for determining minimum inhibitory concentrations (MICs) of widely used antifungals against *Candida* spp., it became possible to evaluate with greater confidence whether in vitro susceptibilities correlated with clinical response to therapy. In order to understand how these issues impact on clinical practice, it is necessary to review the ways in which these drugs work, the molecular mechanisms involved in resistance, and the relationship between increasing use and changing epidemiology of the pathogens involved.

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ANTIFUNGAL DRUGS

The main classes of antifungal agents, their respective members (Table 1) and mechanisms of action[5] are as follows.

Table 1. Main classes of antifungal drugs in clinical use or undergoing evaluation*

Polyenes
Amphotericin B desoxycholate
Liposomal amB ('Ambisome')
AmB lipid complex ('Abelcet')
AmB colloidal dispersion ('Amphocil')
Liposomal nystatin ('Nyotran')*
Azoles
Ketoconazole
Fluconazole
Itraconazole
Voriconazole*
Posaconazole*
Ravuconazole*
Flucytosine
Allylamines
Terbinafine
Echinocandins
Caspofungin*
Anidulafungin*
FK 463*

Polyenes

Amphotericin B (amB) and nystatin are the two clinically useful members of this group. For many years, amB was the only therapeutic agent of value to treat IFI, and because of this, together with its broad spectrum of activity, it acquired the somewhat dubious title of 'gold standard' therapy, despite a significant lack of supporting evidence from randomized clinical trials. AmB frequently causes nephrotoxicity, and this limits its role as an effective agent. The use of nystatin has been restricted to topical administration because of even greater potential for toxicity.

The inhibitory effect of the polyenes results from their interaction with plasma membrane sterols, in particular ergosterol. In the case of amB, this leads to the development of pores or channels, which in turn cause leakage of potassium and other cytoplasmic constituents, with cell death as the outcome. There are now three commercially available lipid-associated formulations of amB, and also liposomal nystatin, which is still undergoing clinical evaluation. These have been shown to cause significantly less nephrotoxicity than the parent desoxycholate formulation. Although they retain the same target of action, the mechanism by which each is delivered and transfers to its target in the fungal cell has not been fully elucidated.

Azoles

The imidazoles clotrimazole, miconazole and ketoconazole, and the newer triazoles fluconazole and itraconazole, are the principal members of this class. Their target is also the plasma membrane, but mainly through inhibition of ergosterol biosynthesis. The triazoles inhibit cytochrome P-450-dependent 14-alpha demethylation of lanosterol, the precursor of ergosterol. The production of this enzyme is regulated by the *ERG11* gene.

Fluconazole has particularly good activity, and proven clinical efficacy, against *Candida albicans* and *Cryptococcus neoformans* but no useful activity against *Aspergillus* spp. Itraconazole has a broader spectrum than fluconazole but its clinical use has been hampered by the lack of a parenteral formulation, which has only recently become available, and also because the oral solution is poorly tolerated. Voriconazole is a new triazole in the advanced stages of clinical evaluation. It has a broad spectrum of activity and looks to be particularly promising for treatment of pulmonary aspergillosis. Although the azoles exhibit mainly fungistatic activity in vitro, they may be fungicidal under certain circumstances in vivo.

Flucytosine (5FC)

This enters fungal cells through the action of a cytosine permease and is converted by cytosine deaminase into the active molecules that inhibit both DNA and RNA synthesis. It is active against both *Candida* spp and *Cryptococcus neoformans*. Its main use is in treatment of cryptococcosis in combination with amB because of superior efficacy over amB when given alone.[2] It may also be used as combination therapy in invasive

Table 2. Antifungal drug MIC breakpoints* and their interpretation

		MIC Category (mg/L)		
Organism	Antifungal	Sensitive	Sensitive dose-dependent	Resistant
Candida spp.	Fluconazole	<8	>8 and ≤32	>32
	Itraconazole	≤0.125	>0.125 and ≤0.5	>0.5
	Flucytosine	≤4	>4 and ≤16	>16
	Amphotericin			>0.5
Cryptococcus neoformans	Fluconazole			≥16
	Flucytosine*	≤4	>4 and ≤16	>16

Adapted from reference no. 33. *Breakpoints not established.
No breakpoints provided for mold susceptibility or amB versus yeasts.

candidiasis,[6] especially in infections that are not responding adequately to the agent of first choice, usually amB, or because of amB toxicity. More recently, there has been interest in combining 5FC with azoles to avoid nephrotoxicity. Flucytosine is rarely used alone because of the likelihood of development of resistance.[7]

Allylamines

These act by inhibiting squaline epoxidase, which is involved in the early stages of ergosterol biosynthesis. The principal member of this group is terbinafine, which is highly active against dermatophytes and has proved more effective than griseofulvin for this indication. Its potential role in treating invasive fungal infections, in combination with other antifungals, is the subject of increasing interest.

Echinocandins

These represent a new class of antifungal agents with a novel mode of action by inhibition of beta-(1,3)-glucan synthase. Caspofungin is the first member of this group to become available for clinical use, having recently been licensed in the USA for salvage therapy of invasive aspergillosis. It is also effective in oropharyngeal candidiasis.

DEFINITIONS OF DRUG RESISTANCE

Because of the previous lack of a standardized methodology for the in vitro determination of antifungal drug susceptibility, clinical failure was often the sole, and inadequate, indicator of drug resistance. Following the development of the reference method for *Candida*,[4] breakpoints have been proposed for fluconazole, itraconazole and 5FC against yeasts which can be used to assess the likelihood of a clinical response to therapy on the basis of the MIC of the strain (Table 2). Using these criteria, most *Candida* spp. show either full or intermediate susceptibility to fluconazole and itraconazole. These MIC categories can be interpreted to mean that a clinical response is very likely with susceptible isolates or will be achieved with a higher dose when the MIC falls in the 'dose-dependent' category. The exception is *Candida krusei*, because it is regarded as being inherently resistant to fluconazole.

In vitro susceptibility testing may reveal that a clinical isolate is resistant to an antifungal even though the patient had never previously been treated with that drug; this is primary or intrinsic resistance. Fungi that may, with varying frequency, demonstrate primary resistance to the main antifungal drugs are listed in Table 3.

Alternatively, a fungal strain may be observed to become resistant following a course of antifungal treatment, and this represents secondary or acquired resistance. To confirm this, it is necessary to test both the pre- and post-treatment isolates together, using the same susceptibility method.

MECHANISMS OF ANTIFUNGAL DRUG RESISTANCE

Azole resistance

Azole resistance in *Candida* spp. has now been the subject of detailed investigation and review.[5,8,9] The principal mechanisms are as follows.

Alterations in the target lanosterol demethylase
This is due to mutation(s) in the *ERG11* gene resulting in reduced affinity of the target for the azole. This mechanism appears to account, in part at least, for the intrinsic resistance of *Candida krusei* to fluconazole.[10] Several point mutations have been documented when fluconazole-sensitive and -resistant isolates of *Candida albicans* have been investigated.[11] Point mutations in the *ERG11* gene have been reported in azole-resistant isolates.[12] Over-expression of lanosterol demethylase has been observed in *Candida glabrata* associated with resistance to both fluconazole and itraconazole, but this may be a less important mechanism overall.[13] Alterations can occur due to mutations in other *ERG* genes, and may additionally be involved in resistance of *Cryptococcus neoformans* to azoles.[14]

Table 3. Fungi that may be inherently resistant to the main antifungal agents

Antifungal		
Amphotericin B	*Fluconazole*	*Flucytosine*
Trichosporon beigelii	*Candida dubliniensis*	*Candida albicans*
Candida lusitaniae	*Candida krusei*	Non-*albicans Candida* spp.
Aspergillus terreus	*Candida glabrata*	*Cryptococcus neoformans*
Scedosporium apiospermum	*Candida inconspicua*	*Aspergillus* spp.
Scopulariopsis dematiaceous	*Candida norvegensis*	*Dimorphic fungi*
Fusarium spp.	*Aspergillus* spp.	

An energy-dependent efflux mechanism
This causes decreased intracellular accumulation of azoles. There are two types of efflux system that have been identified. These are the ATP binding cassette transporters (ABCTs) and major facilitators (MFs) respectively. The *CDR* genes are important members of the ABCT gene family. Over-expression of these genes is associated with resistance to azoles and also other antifungals. Over-expression of the MF gene *MDR1* (*BEN*^{r}) in *Candida albicans*[15] is associated specifically with fluconazole resistance but not cross-resistance to other azoles. The development of azole resistance in clinical isolates of *Candida* spp. typically manifests itself following prolonged exposure to the drug and may involve several of the above resistance mechanisms concurrently.[16]

Alterations in membrane sterols
This may be another mechanism of azole resistance but it is poorly characterized and an uncommon occurrence to date.

Polyene resistance

Mechanisms of polyene resistance are less well studied than is the case for azoles. This is because amB resistance in clinical isolates is uncommon. One explanation for polyene resistance may be reduced ergosterol content in the fungal cell membrane. The ergosterol is replaced by other sterols that have reduced affinity for the polyene. The genetic mechanisms involved have not been comprehensively investigated.

Flucytosine resistance

Flucytosine resistance in *Candida albicans* or *Cryptococcus neoformans* is most commonly due to mutational changes in cytosine deaminase or uracilphosphoribosyltransferase, which are involved in the pyrimidine salvage pathway.

Table 4. Some risk factors, other than drug resistance, for failure of systemic antifungal therapy

Risk factor	*Example*
Impaired drug absorption	Itraconazole capsules used in neutropenic patients
Accelerated drug metabolism	Azoles by rifampicin
Poor penetration into site of infection	amB in meningitis/brain infection
Antagonism	Azole-reducing effect of amB
High fungal burden	OPC in HIV-positive patients
Foreign body is source of infection	Central line in candidemia
Persistent immunocompromise	Hepatosplenic candidiasis

Allylamine resistance

Although resistance to terbinafine appears to be rare in clinical yeast isolates, it has been shown that some azole-resistant strains which over-express either *CDR1* or *MDR1* are cross-resistant to terbinafine.[17]

Echinocandin resistance

Echinocandin resistance has not been investigated in any detail, because of insufficient clinical experience. The reduced activity of caspofungin against *Cryptococcus neoformans* may be the result of lower affinity for the target glucan synthase enzyme.[18] There is no evidence that strains of *Candida* spp. that are resistant to several azoles are cross-resistant to caspofungin, and this would suggest that efflux pumps do not impair the activity of this new drug.

CHANGING EPIDEMIOLOGY OF FUNGAL INFECTIONS

Candida spp., *Aspergillus* spp. and *Cryptococcus neoformans* are the principal causes of IFI, although there is an increasing number of infections due to 'emerging' fungal pathogens, *Fusarium* and *Scedosporium apiospermum* being two notable examples. While *Candida albicans* is recognized as being the most frequent cause of candidiasis, there are recent reports that suggest a 'pathogen shift'. To illustrate this. a comparison between the *Candida* species documented in two studies of invasive candidiasis, published in 1993 and 1998, reveals that the proportion of *Candida albicans* infections fell from over 70% to less than 60%, while the incidence of infections due to non-*albicans Candida* species almost doubled.[19,20] In the latter study,[20] in vitro antifungal susceptibility testing, performed according to the NCCLS recommendations,[4] showed that while isolates of *Candida albicans, Candida parapsilosis* and *Candida tropicalis* were almost universally susceptible to fluconazole and itraconazole, the resistance rates of *Candida glabrata* and *Candida krusei* were 8% and 100% respectively. In neutropenic patients who receive azole prophylaxis, *Candida glabrata* and *Candida krusei* are the predominant yeast flora and the main cause of candidemia.[21]

RISK FACTORS FOR FAILURE OF ANTIFUNGAL THERAPY

When antifungal therapy fails, the possibility of either primary or acquired drug resistance of the organism has to be considered. However, there are other factors that relate to both the drug and the infected host which may also play a significant role, and these have to be taken into consideration (see Table 4). When treatment is administered orally, there is the possibility of impaired absorption and inadequate serum/tissue drug concen-

trations causing treatment failure. This has been reported when itraconazole capsules have been given to neutropenic patients. A further problem of azole use is reduced efficacy due to accelerated metabolism when given together with other drugs that induce hepatic cytochrome P450 enzymes, the most common example being rifampicin.

The pharmacokinetic profile of the drug also needs to be taken into consideration. AmB penetrates the blood–brain barrier poorly, while 5FC has excellent penetration; this may explain the improved outcome of cryptococcal meningitis when these two agents are used in combination compared to amB use alone.

It is clear that the immune status of the infected host is critical to the outcome of an invasive fungal infection. Risk factors for acquired fluconazole resistance in HIV patients include $CD4^+$ lymphocyte count of <50 cells/mm^3, indicative of advanced AIDS, recurrent episodes of OPC, and prolonged prior exposure to azoles.[22,23] In the case of neutropenic patients, invasive aspergillosis often fails to respond to antifungal agents in the face of continuing bone marrow failure. Patients who have made a recent recovery from neutropenia, and who develop the uncommon condition chronic hepatic candidiasis, are often unresponsive to antifungal drugs but may respond when they are combined with immunotherapeutic agents.[24] The presence of vascular catheters is now recognized to be a major risk factor for the development of candidemia. Their presence also increases the likelihood of persistence of the infection despite adequate treatment.[25] The development of *Candida* biofilms coating the lumen of the catheter appears to enable the organism to persist and resist the action of antifungal drugs.[26]

CLINICAL IMPACT OF ANTIFUNGAL DRUG RESISTANCE

One of the earliest reports of acquired resistance to a systemic azole antifungal concerned a cohort of patients suffering from the rare inherited condition chronic mucocutaneous candidiasis.[27] Twenty-one patients had received ketoconazole for prolonged courses of up to 2 years. Two patients who relapsed on ketoconazole yielded *Candida* isolates with MICs >100 mg/L. Most cases of acquired resistance to azoles are HIV-positive patients being treated for OPC. Azoles quickly became established as the treatment of first choice, and fluconazole has been extensively used for this indication. Since the late 1980s, there have been reports of both mycological and clinical failure with fluconazole, including cases where, despite an initial response to therapy, there was subsequently no response to escalating doses up to 800 mg/day, thereby suggesting the development of drug resistance. Typically, resistant strains of *Candida albicans* are isolated from patients with unresponsive infections. Barchiesi et al[28] matched the MICs of isolates with outcome of infection in AIDS patients and showed that there was a good correlation between in vitro resistance and failure of treatment. Typing of repeat isolates is important to confirm that the same strain is involved. Another explanation for clinical failure is a new infection with a fluconazole-resistant non-*albicans Candida* species such as *Candida glabrata* or *Candida krusei*. These species may alternatively only colonize the oropharynx or cause co-infection as a result of selective pressure associated with fluconazole use.

In a study of deep-seated candidiasis due to *Candida albicans* or other *Candida* spp. in HIV-negative patients, MICs corresponded well with the outcome of treatment.[29] However, when Rex et al[30] studied 100 bloodstream isolates stored from an earlier randomized treatment trial, they found that in 16 cases where the MIC indicated susceptibility, the infection failed to respond to fluconazole, while in four there was clinical success where the MIC was >32 mg/L. In the clinical trial, removal of central vascular catheters had a significant beneficial influence on the outcome of treatment,[25] and how these were managed may therefore have accounted for the incomplete correspondence between in vitro and clinical results.

Fluconazole is available as an over-the-counter drug for treatment of vaginal candidiasis, and in the expectation of its widespread use there has been concern that resistant *Candida albicans* would be reported in this setting. The first such report was in a 38-year-old woman who had persistent symptoms of vaginitis over a 3-month period.[31] *Candida albicans* was isolated with an MIC to fluconazole of >40 mg/L and cross-resistance to itraconazole. Despite this report, the isolation of fluconazole-resistant *Candida albicans* in HIV-negative individuals in the community is a rare event.[32]

Correlations between itraconazole MICs and clinical outcome appear to be less good than for fluconazole, and this may be because of the bioavailabilty issues surrounding its oral administration.[33] There are no relevant data on the intravenous formulation relevant to this aspect so far.

Developing breakpoints for amB in order to separate susceptible and resistant *Candida* spp. is made difficult by virtue of the fact that the MIC range within which these isolates fall is quite narrow. Variable results are obtained according to the method used. Generally, however, higher MICs correspond with poorer response to treatment. For example, Powderly et al[34] studied 26 cases of candidemia in BMT patients and found that the incidence of fatal infection was significantly greater where the MIC of the isolate was >0.8 mg/L.

In a study of cryptococcal meningitis[35] in which fluconazole was given, either alone or in combination with 5FC, there was no correlation between response to fluconazole and MIC using the reference method; however, when a modified MIC method was used, it was found that treatment failure was significantly associated with higher MICs. No correlation was found between MIC and response to 5FC. The situation is much less

clear for molds. Generally, there is no standardized method for MIC determination that has been clinically validated, although the NCCLS has proposed a broth microdilution method.[36] In vitro resistance in *A. fumigatus* appears to be rare, but when reported it has been shown that in vitro and in vivo findings correspond.[37]

CONCLUSION AND FUTURE PERSPECTIVES

Over the past decade there has been a considerable increase in our knowledge of the mechanisms by which fungi can develop resistance to commonly used systemic antifungal drugs. With the help of the NCCLS method of in vitro susceptibility testing, it has become possible to develop breakpoints for several drugs that can aid in predicting the likely clinical response in infections due to the most frequently pathogenic *Candida* spp. In vitro resistance will often reliably predict clinical failure, whereas susceptibility may not reliably predict clinical success.[33] Other factors relating to the host and the drug itself will often play an important role in determining outcome, and these need to be included in the clinical assessment of the patient.

Microbiology laboratories should identify all *Candida* isolates from deep sites to species level. With the exception of isolates from HIV patients, *Candida albicans* can be predicted to be susceptible to fluconazole and itraconazole, but when there is failure to respond to either of these drugs, or relapse of an infection, the isolate should be tested for susceptibility. It is also important to identify molds to species level in view of the possibility of isolating a strain with inherent resistance. However, no recommendation can be made for the interpretation of MICs for molds, and in particular *Aspergillus* spp., and therefore MIC results should be interpreted with caution.

The marketing of new antifungal drugs, notably voriconazole and caspofungin, will necessitate study of their susceptibility in vitro so that clinical correlates can be established and resistance rates can be monitored. Furthermore, it is likely that one or more of these will be used in combination with existing antifungals, and this will require in vitro studies of these interactions.

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