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Mechanisms of Fungal Resistance
An Overview

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Contents

Abstract

The increased use of antifungal agents in recent years has resulted in the development of resistance to these drugs. The significant clinical implication of resistance has led to heightened interest in the study of antifungal resistance from different angles. In this article we discuss antifungal susceptibility testing, the mode of action of antifungals and mechanisms of resistance.

Antifungals are grouped into five groups on the basis of their site of action: (i) azoles, which inhibit the synthesis of ergosterol (the main fungal sterol); (ii) polyenes, which bind to fungal membrane sterol, resulting in the formation of aqueous pores through which essential cytoplasmic materials leak out; (iii) allylamines, which block ergosterol biosynthesis, leading to accumulation of squalene (which is toxic to the cells); (iv) candins (inhibitors of the fungal cell wall), which function by inhibiting the synthesis of β 1,3-glucan (the major structural polymer of the cell wall); and (v) flucytosine, which inhibits macromolecular synthesis.

Different mechanisms contribute to the resistance of antifungal agents. These mechanisms include modification of *ERG11* gene at the molecular level (gene mutation, conversion and overexpression), over expression of specific drug efflux

pumps, alteration in sterol biosynthesis, and reduction in the intracellular concentration of target enzymes. Approaches to prevent and control the emergence of antifungal resistance include prudent use of antifungals, treatment with the appropriate antifungal and conducting surveillance studies to determine the frequency of resistance.

The increased incidence of fungal infections, particularly in patients with impaired immune function, has summoned the need for more effective antifungals to replace many of the existing agents, which are not optimal against emerging fungal infections, exhibit host toxicity or have a high propensity to induce the development of microbial resistance. The major contributing factors for the development of fungal resistance are considered to be the extensive and prolonged use of antifungal agents. For example, resistance to fluconazole is especially common in patients infected with HIV who require long-term, prophylactic therapy to prevent a variety of opportunistic fungal infections to which they are susceptible. Indeed, in one investigation, 33% of patients with AIDS were found to have fluconazole-resistant strains of *Candida albicans* in their oral cavities.[1] Nonetheless, the good safety profile, bioavailability and clinical effectiveness of fluconazole has led to its continued use in patients with cancer and neutropenia, and in bone marrow transplant recipients.

The significant clinical ramifications of antifungal resistance have led to heightened interest and concern. Current research efforts in this area are directed at identifying the molecular mechanisms responsible for resistance, developing more effective drugs and improving methods to detect resistance when it occurs. Although the results of this work have substantially increased our understanding of fungal resistance, especially at the molecular level, more remain to be addressed. The apparent fact that fungal resistance mechanisms will constantly evolve in response to the use of new drugs highlights the importance of identifying new resistance genes, developing safer and more effective drugs, and implementing novel strategies to detect, treat and prevent infections caused by resistant fungi.

The past decade has witnessed a significant increase in the number of pathogenic fungi exhibiting resistance to antifungal agents. Such resistance has important implications for morbidity, mortality and healthcare costs in hospitals, as well as in the community at large.

The study of antifungal resistance has lagged behind that of antibacterial resistance for several reasons. Perhaps most importantly, fungi were not considered as important pathogens until relatively recently.[2,3] For example, the annual death rate as a result of candidiasis remained constant from 1950 to about 1970. Since 1970, this rate increased dramatically in conjunction with the frequent and often indiscriminate use of broad-spectrum antibacterial agents, the common use of indwelling intravenous devices and the rise in the number of immunocompromised individuals as a result of advances in cancer treatment and the spread of AIDS.[4] These developments and the associated increase in fungal infections[5,6] have intensified the search for new, more efficacious agents with improved safety profiles to combat serious fungal infections.

For nearly 30 years, amphotericin B was the sole drug available for the treatment of serious fungal infections. Although amphotericin B exhibits superior clinical effectiveness, relative to azole antifungals in the treatment of systemic candidiasis, its narrow therapeutic index and significant nephrotoxicity has limited the overall utility of this drug. The approval of the imidazole- and triazole-based antifungals in late 1980s and early 1990s was an important step that greatly advanced the ability to safely and effectively treat local and systemic fungal infections. The high safety profile of the triazoles, particularly fluconazole, led to their extensive, sometimes prophylactic, use. Indeed, since its launch, fluconazole has been used to treat in excess

of 16 million patients, including over 300 000 patients with AIDS in the US alone.[7-9] As expected, the extensive use of fluconazole has resulted in the development of resistance, particularly in this AIDS population as described in section 3.

Impressive strides have been made in elucidating the molecular basis of antifungal drug resistance, especially in the last 5 years. This review provides an update on antifungal resistance mechanisms with brief comments on clinical relevance. The aim is to provide an understanding of fungal resistance mechanisms that is accessible to clinicians who prescribe antifungal drugs, and members of the scientific community who may wish to study them in the future.

1. Antifungal Susceptibility Testing

Initially, the concept of fungal minimum inhibitory concentration (MIC) testing was irrelevant because no alternative to amphotericin B existed. With the introduction of flucytosine (5-FC) in the 1970s and the azoles in the 1980s, the concept of MIC testing became timely as an aid to selecting the most appropriate drug. At the beginning of the 21st century, the growing significance of fungal disease, the expanding availability of antifungal drugs and the development of fungal resistance, make the need for relevant MIC data urgent.

In 1983, the National Committee for Clinical Laboratory Standards (NCCLS) established a subcommittee to standardise fungal MIC determination. Rex et al.[10] summarise the complexity of such standardisation by pointing out that variations in inoculum size and preparation, incubation time and temperature, media, and endpoint determination can cause MIC determinations to vary more than 50 000-fold. Multiple groups of researchers worldwide were challenged to agree on standards that would generate reproducible MIC data in the range of normal serum drug concentrations and were sensitive enough to detect organisms with truly different drug susceptibilities. As a result of this work, in 1997 the NCCLS adopted the M27 protocol for the susceptibility testing of yeasts.[11]

Despite its adoption by the NCCLS, M27 endpoints can be difficult to interpret for some drugs. For amphotericin B, there is a sharp transition from visible growth to no visible growth at the MIC and the endpoint is readily apparent. For the azoles, in particular, there is a prominent trailing effect, which results in growth at all drug concentrations regardless of susceptibility. Therefore, determination of the MIC depends on a difficult visual assessment of 50 to 80% reduction in growth relative to the drug-free control.

Furthermore, the relevance, or pharmacodynamic correlate, of fungal MIC values is not yet established. The concept of an MIC has proven useful in guiding antibacterial therapy; however, it is more accurate to think of the MIC as a predictor of failure rather than of success. Recovery from an infection is dependent on many patient-, drug- and organism-related factors, of which the MIC is only one.

The ability of MIC values to predict antifungal therapeutic failure is far from universal and it is critical to remember this when evaluating *in vitro* antifungal susceptibility data. NCCLS has established interpretive breakpoints for fluconazole, itraconazole and flucytosine (table I) by examining all pertinent animal and human data, and attempting to define an MIC above which therapeutic failure with that drug is likely.[12] It is important to emphasise the relatively arbitrary nature of all breakpoint determinations and the absence of controlled, evaluative prospective trials. The existent

Table I. Tentative interpretive guidelines for susceptibility testing *in vitro* of *Candida* species

Antifungal agent	Susceptible	Susceptible-dose dependent[a]	Resistant
Fluconazole	≤8	16-32	≥64
Itraconazole	≤0.125	0.25-0.5	≥1
Flucytosine	≤4	8-16	≥16

a For flucytosine, the old term 'intermediate susceptibility' is used by the NCCLS.

breakpoint data correlate most strongly for oesophageal candidiasis in patients with HIV, and must be interpreted cautiously in other clinical scenarios since the prediction of therapeutic efficacy based on an MIC is supported by fewer data.[13] Now that the interpretive breakpoints for antifungal susceptibility of *Candida* spp. are available, the Mycosis Study Group has recommended their use in the management of patients with candidaemia. Routine antifungal susceptibility testing should not be performed and should be reserved for those not responding to therapy, and to infections by non-albicans species. (e.g. *Candida glabrata*).

For amphotericin B, some investigators have begun to use an MIC cut-off of <1 mg/L as susceptible but this is on the basis of one study, the results of which have not been reproduced.[14] Indeed, a major limitation seems to be a clustering of nearly all M27 MIC determinations for amphotericin B around 0.5 to 1 mg/L, suggesting that this protocol may be relatively insensitive for amphotericin B, despite its clear endpoint.[13]

MIC determination and interpretation against filamentous fungi such as *Aspergillus* spp. lags even further behind that of yeasts. Currently, the NCCLS has proposed the M38-P protocol for MIC determination against filamentous fungi.[15] It is essentially a variation of the M27 protocol but has not yet been successful in generating clinically useful MIC values.[16] This delay may be attributed to difficulty establishing reliable endpoints that determine the MIC, and the low numbers of patients from whom it is easy to diagnose infection and to culture such organisms.

The development of the new class of echinocandin antifungals raises new issues in susceptibility testing. Data is accumulating to show that the developed NCCLS methodologies are not suitable for measuring the antifungal susceptibility of these agents. Consequently, several investigators are attempting to develop alternative assays that may be useful for susceptibility testing of fungi to echinocandins. Recently our group used a 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2H-tetrazolium hydroxide (XTT)-based assay to evaluate the effects of mulundocandin (an echinocandin-like compound) on *Aspergillus fumigatus in vitro* and compared this technique with the microdilution assay performed by the NCCLS M38-P method. Our data showed that, in contrast to the NCCLS methodology, which does not predict the activity *in vivo*, the XTT-based assay showed that *A. fumigatus* is susceptible to mulundocandin. This indicates that the XTT-based assay might be useful for determination of the susceptibilities of moulds to echinocandin.[17]

2. Mechanisms of Antifungal Action and Resistance

2.1 Azole-Based Antifungal Agents

The azoles, including the imidazoles (ketoconazole and miconazole) and the triazoles (fluconazole, itraconazole, voriconazole, posaconazole and ravuconazole) function by inhibiting lanosterol 14α-demethylase, a key, cytochrome P450 (CYP)-dependent enzyme in the ergosterol biosynthetic pathway that participates in the multistep conversion of lanosterol to ergosterol (figure 1). Ergosterol is a necessary sterol important for maintaining the structural integrity of the fungal cell membrane.[18] Inhibition of 14α-demethylase leads to depletion of ergosterol, which leads to the formation of membranes with altered structure and function, and accumulation of sterol precursors, especially 14α-methyl fecosterol and 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol. Accumulation of the latter diol has been associated with growth arrest in *Saccharomyces cerevisiae* and *C. albicans*.[19-21] Although azoles are usually effective against different *Candida* species, they tend to be less active against the emerging pathogen *Candida krusei* (although the new triazoles, e.g. voriconazole, have potent activity against this species).

Mammalian cholesterol biosynthesis is also affected by azoles at the stage of 14α-demethylation; however, the dose required to produce the same degree of inhibition is much higher than that required for fungi.[22-24] Human sterol biosynthesis is most prominently effected by ketoconazole.

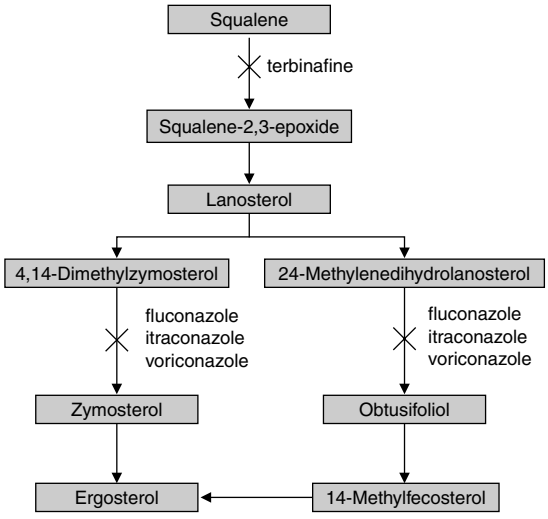

Fig. 1. Ergosterol biosynthetic pathway in fungi.[8]

Buttke and Chapman[25] showed that ketoconazole inhibited the incorporation of ^{14}C acetate into cholesterol, with a resultant accumulation of ^{14}C lanosterol. Importantly, this inhibition was affected by drug concentrations obtained therapeutically. Another study reported that ketoconazole specifically inhibited the intracellular transport of low-density lipoprotein cholesterol. In addition, ketoconazole also had a general effect on cholesterol movement.[26] The potent ability of ketoconazole to inhibit mammalian cholesterol could, in part, explain the high toxicity of this azole, which limits its clinical utility.

2.1.1 Modification of the ERG11 *Gene at the Molecular Level*

The gene encoding 14α-demethylase is currently referred to as *ERG11* in all fungi from which it has been cloned. Mutation, gene conversion and overexpression of *ERG11* have been investigated to determine if these genetic modifications can contribute to the development of antifungal resistance.

Analysis of a series of *C. albicans* clinical isolates (17 strains obtained from the same patient over a 2-year period) described by Redding et al.,[27] using biochemical and molecular techniques showed that a dramatic decrease in fluconazole susceptibility occurred in isolate 13.[28] Comparison of the DNA sequences of *ERG11* homologs from this azole-resistant isolate and a sensitive *C. albicans* strain revealed a point mutation (R467K) in the resistant isolate that results in the replacement of arginine (R) for lysine (K) at position 467.[29] Since this mutation is in close proximity to a cysteine that participates in the coordination of the iron atom in the heme cofactor of the enzyme,[30] it has been suggested that this mutation causes structural or functional changes associated with the heme.[31] Preliminary studies indicate that R467K by itself can confer azole resistance by reducing the affinity of the enzyme for fluconazole.[32]

A similar point mutation T315A, in which threonine (T) was replaced with alanine (A) at position 315, was generated in the *ERG11* gene from a laboratory strain of *C. albicans*.[33] This particular mutation was selected on the basis of the architecture of the active site of the enzyme, which is positioned directly above the heme cofactor.[30] Studies of this mutated version of the *C. albicans ERG11* gene in the genetically tractable yeast *Saccharomyces cerevisiae* showed that its altered enzyme product was less active and had a reduced affinity for fluconazole.

Other investigators have reported additional mutations that play a role in azole resistance. Recently Edlind et al.[34] cloned and sequenced the *A. fumigatus* CYP sterol 14α-demethylase (*CYP51*) gene and reported that the resistance of this organism to azoles may be due to mutation of Ile 301 residue, which corresponds to *C. albicans* Thr 315 residue, to an Ala residue in resistant strains.

Existing exclusively as a diploid organism, *C. albicans* harbours two copies of each gene. Allelic differences between copies of a gene are common among clinical *C. albicans* isolates. The ability of *C. albicans* to preferentially replace one allele of a gene for another may contribute to or enhance azole resistance. Indeed, analysis of *ERG11* in resistant isolates showed that all allelic differences present in sensitive strains were absent and that both copies contained the R467K mutation.[29] Re-

sistant strains harbouring two copies of *ERG11* containing the R467K mutation are more resistant to azoles compared with strains with a single mutated allele.[31] Since portions of the gene encoding homoserine kinase (*THR1*), which is positioned immediately downstream of *ERG11*, also lacked allelic variation, this suggests that the gene conversion event resulted in the loss of allelic variation at the *ERG11* locus.

Overexpression of *ERG11* has been documented in clinical isolates of *C. glabrata* and *C. albicans*.[28,35] In *C. albicans* isolates exhibiting this phenotype, the level of *ERG11* expression was only increased 5-fold.[28,31,32] Furthermore, other resistance mechanisms, such as the R467K mutation and overexpression of genes encoding drug efflux pumps (see section 2.1.2) are invariably present in strains that overexpress *ERG11*. Thus, the contribution of *ERG11* overexpression to azole resistance is not clear. The scarcity of clinical isolates in which overexpression of *ERG11* has been observed, together with the finding that other resistance mechanisms may be operative in the same strain, suggests that overexpression of *ERG11* plays only a limited role in clinical resistance to azoles. Azole-resistant *C. albicans* and *Cryptococcus neoformans* clinical isolates may also originate as a result of mutation to other genes in the ergosterol biosynthetic pathway, namely *ERG2* and *ERG3*.[20,36,37] These genes encode enzymes (C-8 sterol isomerase and C-5 sterol desaturase, respectively) that function downstream of *ERG11*. As described in section 2.1, inhibition of 14α-demethylase results in the accumulation of the diol that arrests fungal growth. *C. albicans erg3* strains that continue to grow in the presence of 14α-demethylase inhibitors have been shown to do so by blocking the synthesis of the toxic diol, presumably by the activity of the defective C-5 sterol desaturase.[20]

2.1.2 Drug Efflux

Evidence implicating drug efflux as a mechanism of resistance in *Candida* species continues to mount. This mechanism is believed to be the prominent mechanism responsible for the resistance phenotype observed in clinical isolates. Parkinson et al.[38] compared pre-treatment (azole-susceptible) and post-treatment (azole-resistant) isolates of *C. glabrata* and showed that the post-treatment isolate accumulated less fluconazole than the susceptible one. The reduced ability of the resistant strain to accumulate fluconazole was a consequence of energy-dependent drug efflux.[38] In an extension of these studies, Hitchcock and coworkers examined the mechanism of resistance to azoles in *C. albicans*, *C. glabrata* and *C. krusei* using the fluorescent dye rhodamine 123 (Rh123), which is known to be transported by a number of organisms displaying multi-drug resistance (MDR).[39] Their results showed that azole-resistant strains accumulated less Rh123 than did azole-susceptible strains. In *C. glabrata,* a single efflux pump appears to be capable of exporting both Rh123 and fluconazole, since accumulation of these drugs in this fungus is competitive. By contrast, no competition is observed in *C. albicans*, suggesting that separate pumps are used for each drug.[39] Drug efflux has recently been observed in a laboratory derived *C. neoformans* strain resistant to azoles and polyenes and in a *A. fumigatus* clinical isolate exhibiting resistance to itraconazole.[40,41]

Fungi possess at least two types of efflux pumps; those belonging to the ATP-binding cassette (ABC) and the major facilitator (MF) superfamilies of proteins. ABC proteins contain four domains, two domains that span the membrane, and as their name indicates, two nucleotide binding domains (NBD) specific for ATP.[31] The only exception that has been observed is in members of the YEF3 subfamily, which lack membrane-spanning domains.[37] The MF efflux pumps are associated with fluconazole resistance.[31]

The availability of the complete sequence of the genome of *S. cerevisiae* has allowed the number of candidate ABC and MF genes to be estimated in this model yeast. Thirty genes that encode proteins with ABCs and 28 genes that encode MF efflux pumps were identified.[42] The 30 candidate ABC genes were grouped into six subfamilies (PDR5, ALDP, CFTR/MRP, MDR, YEF3, and RLI) on the basis of phylogenetic analyses. However, only the

PDR5, CFTR/MRP and MDR subfamilies contain genes that encode proteins known to confer azole resistance.[31]

Efflux pumps belonging to the ABC superfamily in *C. albicans* and more recently *C. glabrata*[43] and *Aspergillus nidulans*[44] continue to be identified.[43,45] However, the most notable ABC efflux pumps in *Candida* spp. are encoded by members of the PDR5 subfamily. These genes have been named *CDR* for *Candida d*rug *r*esistance, and are the only ones characterised thus far that are associated with azole resistance. The results of several molecular and genetic studies indicate that at least 10 *CDR* genes exist in the *C. albicans* genome.[32,46]

The first *CDR* gene (*CDR1*) was cloned by complementation of a *S. cerevisiae pdr5* mutant exhibiting hypersensitivity to azole antifungals, cycloheximide (cyclohexamide) and chloramphenicol.[47] *CDR1* encodes a transporter (Cdr1) with homology to the human P-glycoprotein, a MDR pump overexpressed in neoplastic cells resistant to a number of structurally and functionally distinct chemotherapeutic agents.[48] Evidence linking *CDR1* with azole resistance was obtained from the molecular analysis of 16 sequential *C. albicans* isolates cultured from five patients with AIDS.[49] The strains were selected on the basis of increasing fluconazole resistance following prolonged treatment with the drug. In some resistant strains, decreased accumulation of fluconazole was associated with up to a 10-fold increase in the mRNA levels of the *CDR1* gene. Other resistant isolates overexpressed the gene encoding Ca*MDR1*, a MF pump, while maintaining normal levels of *CDR1* mRNA.

Additional, independent investigations have also shown that CDR1 expression is increased in azole-resistant strains compared with azole-sensitive ones.[28,50] *CDR2* was also cloned by complementation of the *S. cerevisiae pdr5* mutant phenotype.[45] The amino acid sequence of the protein (Cdr2) encoded by this gene is 84% identical to that of the Cdr1p. *CDR2* is associated with resistance to azoles, terbinafine and amorolfine. The concomitant overexpression of *CDR1* and CDR2 has been documented in two azole-resistant, clinical isolates compared to matched sensitive strains. However, in one of the resistant strains the level *CDR2* overexpression was 6-fold greater than that of *CDR1*, whereas the mRNA levels for the two genes were nearly identical in the other resistant strain. Dual overexpression of *CDR1* and *CDR2* suggests that these genes may share a common transcriptional regulator, possibly *CAP1*, the *C. albicans* homolog of a *S. cerevisiae* gene (y*AP1*) that encodes a protein known to regulate expression of genes in the PDR subfamily.[51]

Until the recent cloning of *FLU1* the only known gene encoding a MF transporter in pathogenic fungi was *CaMDR1* (formerly referred to a *BEN*^{r}) from *C. albicans*.[52,53] The *CaMDR1* gene was cloned by exploiting its ability to confer benomyl (a fungicide) and methotrexate resistance to *S. cerevisiae*, which is normally sensitive to these drugs.[54] The results of several, unrelated investigations showed that *CaMDR1* was dramatically overexpressed in some azole-resistant *C. albicans* strains.[49,50] The fact that *CDR1* expression was normal in these resistant strains suggests that increased expression of *CaMDR1* is associated with azole resistance. *CaMDR1* exhibits selectivity among the azoles for overexpression of the gene in *S. cerevisiae*, resulting in cells that became resistant to fluconazole but not to ketoconazole or itraconazole, whereas the products of *CDR* genes export several types of azole drugs.[51,52] Analysis of the amino acid sequence predicted by *FLU1* revealed that it exhibits significant homology to the *CaMDR1* gene product. Although *FLU1* was cloned by its ability to complement a *S. cerevisiae* strain hypersusceptible to fluconazole, this gene does not appear to be necessary for the development of fluconazole resistance in clinical *C. albicans* isolates.[53] In fact, the protein encoded by this gene may exhibit higher substrate specificity towards mycophenolic acid than for fluconazole.[53]

Although genetic experiments have shown that each of the mechanisms described above can individually confer azole resistance in laboratory

strains of *C. albicans*, it is unlikely that highly resistant clinical isolates are generated as the result of single genetic events. Evidence in support of this notion has come from the molecular analysis of a series of 17 *C. albicans* isolates cultured from a patient infected with HIV.[27]

The patient experienced 15 relapses of oropharyngeal candidiasis over a 2-year period. The level of fluconazole resistance displayed by the isolates steadily increased over the course of therapy and correlated with the dosages administered. Analysis of *CaMDR1*, *CDR1* and *ERG11* mRNA levels in these isolates revealed that *CaMDR1* expression increased dramatically in isolates 2 and 3 and continued to be expressed at levels approximately 25-fold higher than normal in the remaining isolates. *ERG11* overexpression was first observed in isolate 13 and remained at levels approximately 5-fold higher than normal through isolate 17. However, as noted earlier, the increase in *ERG11* expression was accompanied by the R467K point mutation, as well as loss of allelic variation at the *ERG11* genetic locus.[29] The level of *CDR1* expression did not vary in isolates 1 to 15 but was dramatically increased in isolates 16 and 17. Increases in *CaMDR1* expression were directly correlated with increased resistance to fluconazole but had no effect on the cross-resistance of the isolates to itraconazole, ketoconazole or amphotericin B. By contrast, overexpression of *ERG11* and *CDR1* were associated with increased cross-resistance to ketoconazole and itraconazole but not amphotericin B.

These data demonstrate that high level azole resistance, at least in this series of isolates, results from the combined effects of many genetic events. They also suggest that prolonged exposure of a strain to one azole may lead to the development of cross-resistance to other azoles.

Recently, Bouchara et al.[55] described a new mechanism of azole resistance. These authors identified *C. glabrata* isolates that grow as small colonies (petite) and exhibit sensitivity to amphotericin B and complete resistance to ketoconazole and fluconazole. Analysis of the growth pattern of these petite mutants revealed that they have a respiratory deficiency, which was further confirmed by flow cytometric analysis of the fluorescence of Rh123-stained yeast cells. Additionally, polymerase chain reaction amplification of the mtDNA as well as transmission electron microscopy, suggested a partial deletion of the mtDNA analogous to that described from rho-petite mutants of *S. cerevisiae*.

Itraconazole is frequently used to treat invasive aspergillosis. Resistance of *A. fumigatus* and *A. nidulans* has been reported.[41,56,57] A number of researchers investigated the mechanism of resistance to *Aspergillus* spp. These studies revealed that at least three different mechanisms might be responsible for conferring resistance. These include: (i) decreased accumulation of itraconazole;[41,58] (ii) alteration in ergosterol content;[41] and (iii) amplification or overexpression of the CYP-dependent C-14 lanosterol α-demethylase.[56]

2.2 Polyenes

From the 1950s until the discovery of the azoles, polyene antifungal agents such as amphotericin B, represented the standard of therapy for systemic fungal infections.[59] Amphotericin B and other polyenes, including nystatin, function essentially by binding to ergosterol. For amphotericin B, it has been proposed that association with sterols results in the formation of aqueous pores[60,61] that perturb membrane structure and polarity. Such disorganisation increases the permeability of the membrane to protons and monovalent cations, and ultimately causes cell death.[62,63] Although amphotericin B maintains a higher affinity for ergosterol compared with other sterols, the host toxicity displayed by this drug is primarily the result of its binding to cholesterol. Amphotericin B has also been implicated as an inducer of phagocytic cells.

Despite more than 30 years of clinical use, resistance to polyene antibiotics is rare, with resistant isolates confined mostly to the less common species of *Candida*, such as *Candida lusitaniae*, *C. glabrata*, and *Candida guillermondii*.[64] It has been proposed that resistance arises as a result of changes, either quantitative or qualitative, in the sterol content of the cells.[65] According to this hy-

pothesis, resistant cells with altered sterol content should bind smaller amounts of polyene than do susceptible cells. Consistent with this notion, the development of polyene resistance in a strain of *C. albicans* was accompanied by a decrease in the ergosterol content of the cells.[66] This decrease was not due to enzymatic degradation of pre-formed ergosterol but to inhibition of its synthesis. Similarly, sterol analysis of 27 polyene-resistant *C. albicans* isolates obtained from patients with neutropenia revealed that these strains had greatly reduced (74% to 85%) ergosterol content.[67] Comparison of two *C. neoformans* (pre- and post-treatment) isolates from a patient with AIDS who failed antifungal therapy revealed that a correlation exists between amphotericin B susceptibility and sterol pattern.[68] The resistant, post-treatment isolate had a defect in C-8 sterol isomerase leading to accumulation of ergosta-5,8,22-dienol, ergosta-8,22-dienol, fecosterol and ergosta-8-enol, with a concomitant depletion of ergosterol, the major sterol present in the susceptible, pre-treatment isolate.

2.3 Allylamines

Similar to the mechanisms of many other currently prescribed antifungals, terbinafine blocks ergosterol biosynthesis by inhibiting squalene epoxidase, an enzyme that catalyses an early step in the ergosterol biosynthetic pathway, namely, the conversion of squalene to squalene epoxide.[69] Interestingly, the resulting accumulation of squalene, rather than the deficiency in ergosterol, is considered to be the primary cause of fungal cell death.[70,71]

Although therapeutic failure has been observed in patients treated with terbinafine, allylamine resistance in association with clinical use of terbinafine and naftifine has not been reported in pathogenic fungi. However, with the increased use of this agent resistance may be expected, since Van den Bossche et al.[35] recently reported a *C. glabrata* strain that became resistant to fluconazole and expressed cross-resistance to terbinafine. In addition, the *CDR* gene products (Cdr1 and Cdr2) appear to be cross-reactive with terbinafine.[72] The machinery to develop resistance to allylamines is therefore already present in some yeast. This is in contrast with the situation in dermatophytes. Our group tested over 2000 clinical and environmental dermatophyte isolates and we were unable to detect any significant resistance (Ghannoum et al. unpublished observations).

2.4 Inhibitors of Fungal Cell Wall Synthesis

The fungal cell wall contains four classes of macromolecules, namely cell wall proteins, chitin, β1,3-glucan and β1,6-glucan. Since these components are not found in mammalian cells, their pathways of biosynthesis have naturally been targeted for drug development.[69]

A number of compounds have been discovered and described over the past 30 years that affect the cell wall of fungi.[73] This review concentrates on glucan synthesis inhibitors only since at least two antifungal agents that belong to this class of compounds are being evaluated in clinical trials [micafungin (FK-463) and anidulafungin (LY-303366)], and one (caspofungin) has been approved by the US Food and Drug Administration for the treatment of refractory and intolerant invasive aspergillosis. Chitin synthesis inhibitors, such as nikkomycins, have been extensively investigated, but no product has as yet been commercially developed.

2.4.1 Inhibitors of Glucan Synthesis

Of the three groups of compounds (aculeacins, echinocandins and papulacandins) that are specific inhibitors of fungal β1,3-glucan synthase, only echinocandin derivatives are being actively pursued in clinical trials to evaluate their safety, tolerability and efficacy against fungal infections.[74-76] Echinocandins are amphophilic lipopeptides that consist of a cyclic peptide core to which one or more lipid chains are attached.[77] Inhibition of β1,3-glucan synthase, as its name implies, prevents the synthesis of β1,3-glucan, the major structural polymer in the cell wall. Depletion of β1,3-glucan results in osmotically unstable cells that eventually lyse.[78]

Since glucan synthesis inhibitors have recently entered clinical use, resistant strains resulting from therapy are not available. Therefore, knowledge of mechanisms of glucan synthesis inhibitor resistance is based mostly on analysis of laboratory-derived *S. cerevisiae* mutants exhibiting echinocandin resistance.[79,80] It was shown that *S. cerevisiae* develops resistance to lipopeptide antimycotic agents via mutations that alter the protein encoded by *FKS1*, which is the main target of the inhibitor and is presumed to be the catalytic component of the fungal cell wall glucan synthase.[81,82]

Whether parallel mechanisms operate in resistant *C. albicans* clinical isolates is still being explored. However, preliminary findings with pneumocandidn-resistant *S. cerevisiae* and *C. albicans* strains suggest that the molecular basis of resistance to lipopeptide compounds is very similar in these two fungi.[79,80]

Echinocandin-like compounds have potent antifungal activity against *C. albican, A. fumigatus,* and *Pneumocystis carinii.* In contrast, this class of antifungals has limited activity against *C. neoformans*. This lack of activity was proposed to be due to abscence of 1,3-β-D-glucan in the cryptococcal cell wall. A recent study by Feldmesser et al.,[83] showed that although 1,3-β-D-glucan is present in the cryptococcal cell wall, albeit to a lesser extent than in *Candida* species. Therefore, absence of 1,3-β-D-glucan *per se* does not explain the relative lack of efficacy of echinocandins and suggests additional mechanism/s. In this respect, Feldmesser et al.,[83] proposed that the relatively low efficacy of caspofungin against *C. neoformans* may result from reduced activity against *C. neoformans* glucan synthase or from yet undiscovered mechanism of action operative in other fungal pathogens but not in *C. neoformans.*

2.5 Compounds Affecting Protein Synthesis and DNA Replication

2.5.1 Flucytosine

Flucytosine is a fluorinated pyrimidine with inhibitory activity against many yeasts, including *Candida* species and *C. neoformans*. Flucytosine enters fungal cells via a cytosine permease and is converted to fluorouracil (5-FU) by the enzyme cytosine deaminase.[84,85] 5-FU is subsequently converted into 5-fluorouridine monophosphate (FUMP) by a reaction catalysed by uracil phosphoribosyl transferase (UPRTase). FUMP is subsequently converted into either 5-fluorouridine triphosphate (FUTP), which ultimately inhibits proteins synthesis, or 5-fluorodeoxyuridine monophosphate (FdUMP), a potent inhibitor of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division.[86,87]

The initial promise of this agent has been diminished by the high prevalence of primary resistance in many fungal species. Although roughly 10% of *C. albicans* clinical isolates exhibit primary resistance to flucytosine, this incidence is lower than that observed for clinical strains of *C. neoformans* and *A. fumigatus*.[68,83] As a result of the unacceptably high levels of inherent resistance, flucytosine is used in combination with other antifungals, such as amphotericin B and fluconazole, and rarely as a single agent. With the introduction of the new antifungals (e.g. voriconazole and caspofungin), it will be interesting to determine the interaction of flucytosine with them and whether any interaction is synergistic.

Elegant genetic studies performed with *C. albicans* suggest that flucytosine resistance is a recessive characteristic determined by the genotype of a particular strain at the genetic locus defining the resistance gene (*FCY*). Thus, *FCY/FCY* strains are susceptible to flucytosine, *FCY/fcy* herterozygotes exhibit partial resistance, and the recessive *fcy/fcy* genotype, which results via mitotic recombination in the heterozygote, results in a highly resistant phenotype.[88] Unlike *C. albicans*, which uses recombinational processes, flucytosine resistance arises in *C. neoformans* as a result of mutations to this haploid yeast. Indeed, it was shown that mutations in either of two *C. neoformans* genes (*FCY1* and *FCY2*) could confer flucytosine resistance.[88]

At the biochemical level, flucytosine resistance in most *C. albicans* clinical isolates is caused by a

defect in either of the two enzymes involved in the pyrimidine salvage pathway, namely UPRTase and cytosine deaminase. Resistance in these mutants is a result of their inability to convert flucytosine into the toxic fluorinated intermediates described in the first paragraph of this section. Since this pathway is not essential, mutant strains lacking these enzyme activities grow normally under conditions in which pyrimidines are synthesised *de novo*.

3. Clinical Implications of Antifungal Resistance

Antifungal resistance is evolving into a clinically important problem in patients requiring long-term prophylactic or who have a history of antifungal use. Moreover, resistance development is impacting the epidemiology of *Candida* infection manifested by a change from the *albicans* to the non-albicans *Candida*.[31] Increasing evidence suggests that resistance to azoles stems from prolonged therapy with prophylactic levels of these drugs, especially fluconazole. However, out of necessity, HIV-infected patients continue to be administered maintenance doses to control recurrent fungal infections, particularly cryptococcal meningitis[31] and oropharyngeal candidiasis.

The majority of patients with AIDS experience one or more fungal infections during their illness, with oropharyngeal candidiasis the most common. Although fluconazole is usually effective in treating oropharyngeal candidiasis, many patients experience relapses or reinfection as a result of incomplete eradication of the infecting yeasts. The subsequent repeated treatment courses ultimately results in subjecting *Candida* spp. to multiple exposures of the drug. Thus, concomitant with the long-term, repeated usage of fluconazole was the emergence of fungal strains exhibiting resistance to this drug as well as cross resistance to other azoles.[89-91] Unlike the situation with candidal bloodstream infections, the incidence of which remains high, the incidence of oesophageal candidiasis in the HIV/AIDS setting has decreased. This decrease could be attributed to the host immune reconstitution brought about by the advent of highly active antiretroviral therapy (HAART). The decrease in oesophageal candidiasis in both adults and children is documented in the study of Kaplan et al.,[92] who investigated the epidemiology of HIV-associated opportunistic infection in the US in the era of HAART. These authors showed among other opportunistic infections, the incidence of oesophageal candidiasis dropped significantly between 1996 and 1997; however, a disturbing trend of increasing incidence was seen between 1997 and 1998. This observation indicates that its prudent to continue following the epidemiology of this and other opportunistic infections, and that its too early to draw firm conclusions regarding such trends. The decrease in the incidence of opportunistic infections, including candidiasis, has been attributed to the restoration of specific immunity.[93,94] Its critical to clarify that the observation that the incidence of opportunistic infections, including candidiasis, is decreasing in patients infected with HIV is limited to developed countries (mainly the US and Western Europe); however, the incidence is still high in developing countries, particularly sub-Saharan Africa where up to 50% of the population are infected with the HIV virus.

3.1 Antifungal Dose Administration

Unfortunately, appropriate dose administration strategies to curtail the incidence of resistance development have not as yet been identified. To address this need, several treatment options, including duration of treatment, drug dose, dose administration schedule (intermittent versus continuous) will need to be evaluated for their effect on resistance development. In addition, it will be important to determine whether the health status of the patient has an impact on antifungal resistance.

With regard to the amount of drug administered, *in vivo* animal models and experience in humans show that treatment with higher doses of fluconazole is associated with a better outcome. For amphotericin B and flucytosine, the dose administered is limited by the toxicity of these agents, but the favourable therapeutic index of fluconazole makes dose escalation an attractive strategy to

overcome partial resistance and improve clinical outcome. In Germany, fluconazole is licensed in adults for up to 800 mg/day. As summarised by Duswald et al.,[95] even higher doses may be more effective. Most of the data is from case reports and non-comparative trials, and highlights the need for good dose-finding trials.

As we better understand the relationship between MIC and therapeutic outcome, the use of high-dose therapy with newer, less toxic antifungal drugs will be tailored to the patient and the organism. This situation is analogous to the use of high-dose amoxicillin to treat *Streptococcus pneumoniae* with intermediate resistance to penicillin.

The strategy of combination drug therapy for bacterial infections is well established. In contrast, although more than 80 studies have been published of various combination therapies against fungal organisms in animal and *in vitro* models, the data in humans is very limited and combination therapy is not routine. It has been suggested that combination therapies that exploit drugs with different mechanisms of action would force cells to acquire at least two simultaneous mutations to become resistant to therapy.[96] Antifungal combinations that have been tested include amphotericin B with flucytosine or an azole, azoles with flucytosine, or an antifungal plus rifampin. The reader is referred to a recent review that summarises current data available for combination drug strategies.[97] However, because of sparse comparative human data, the true clinical effect of prescribing more than one antifungal drug simultaneously is, in general, unknown.

3.2 Development of New Antifungals

The number of new antifungal drugs in development is impressive, especially when one considers that the mainstay of therapy, amphotericin B, has been in use for more than 30 years, and fluconazole was not introduced until the 1980s. A review of the abstracts from the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) during the years 1995 to 1999 reveals submissions describing 51 specific new antifungal compounds. Since a complete description of all promising antifungals currently in development is beyond the scope of this review, the reader is referred to two recent reviews on the subject.[97,98]

The rapid search for next generation antifungals reflects the clear need for new or modified derivatives of currently used agents that avoid the risk of resistance development and also effectively treat infections caused by strains already exhibiting a resistance phenotype. However, the broad substrate specificity displayed by the efflux pumps necessitates that all new investigational compounds be tested as potential substrates so that similar mechanisms of resistance do not develop.[31] Similarly, investigational drugs should also be monitored for their potential to cause cross-resistance to the polyenes, especially amphotericin B.

The discovery of fungal efflux pumps offers the question, can antifungal drugs be combined with an efflux pump inhibitor to increase efficacy? This situation is analogous to the combination of a β-lactam antibiotic with a β-lactamase inhibitor. MC-510027 is an efflux inhibitor that has been shown to decrease the MIC values of three azoles and terbinafine by 1 to 3 orders of magnitude against several species of *Candida*.[99] Despite the lack of clinical data in animals or humans, this strategy clearly has potential, and may undoubtedly form a future part of antifungal therapy.

3.3 Prevention and Control of Antifungal Resistance

Although the emergence of antifungal resistance impacted fluconazole prophylactic use to some extent, strategies to avoid and suppress emergence of antifungal resistance have not been defined. However, approaches analogous to those recommended for antibacterials could be suggested.[100-102] These measures include: (i) prudent use of antifungals; (ii) treatment with the appropriate antifungal (in cases where the aetiological agent is known); and (iii) conducting surveillance studies to determine the true frequency of antifungal resistance. The fact that the majority of genes involved in resistance development have yet to be identified makes it unrealistic to characterise clin-

ical isolates at the molecular level. However, an attractive approach proposed by White et al.[31] is to use molecular techniques to evaluate the overall health of a cell in the presence and absence of a drug. Additionally, improvements to susceptibility testing methods may be helpful in reducing the use of inappropriate antifungals to which the infecting fungi are already resistant. Furthermore standardising protocols, such as the M38-P protocol for filamentous fungi, for other clinically important fungi will be a major advance to the field of susceptibility testing by enhancing our ability to detect resistance when it occurs.

4. Conclusion

The expression of resistance to antifungal agents is the logical and inevitable consequence of using these agents to treat human infections. With increased use and availability of different classes of antifungal agents, it is anticipated that we will see an increasing number and variety of fungal species resistant to these agents. Continued efforts to study the mechanisms of antifungal resistance and the development of experimental systems, in which individual resistance mechanisms can be studied, will be important components of a strategy to limit the emergence of resistance to these agents and to develop safer and more potent compounds for the future.

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