MINIREVIEWS

Stress, Drugs, and Evolution: the Role of Cellular Signaling in Fungal Drug Resistance

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The survival of all organisms depends critically upon interactions with the environment, mediated largely through the action of small molecules. Small molecules can provide the nutrients to support life (195), mediate communication between organisms (25, 76, 238), or exert toxicity that threatens viability (86, 183). The discovery of small molecules with exquisite potency and selective toxicity to microbes revolutionized modern medicine, as exemplified by the archetypal antibiotic penicillin, the first broad-spectrum antibiotic and the most famous fungal secondary metabolite (14, 211). There are relatively few antimicrobial structural scaffolds that are purely synthetic in origin, and humans rely heavily upon many natural products and their derivatives as agrochemicals, antimicrobials, immunosuppressants, and antineoplastic agents (24, 73, 97). Given that small molecules can have profound effects on cellular signaling and are a ubiquitous presence in the environment, it is not surprising that microbes have evolved diverse mechanisms to survive the challenges of exposure to antimicrobial drugs.

Antimicrobials represent one of the many stresses that a microbial pathogen must sense and respond to in order to thrive in harsh environmental conditions. Antimicrobial drug resistance mechanisms that span the kingdoms can largely be classified into two broad categories. The first category includes mechanisms to bypass the effects of the antimicrobial on the cell. Such mechanisms include alterations in the drug target that block drug binding (6, 99, 165, 227) or increased production of multidrug transporters that simply remove the drug from the cell (72, 74, 175, 200, 214). The second category includes mechanisms that allow the cell to cope with drug-induced stress. Such mechanisms include metabolic alterations that minimize the toxicity of the drug, as well as the activation of chaperones and signal transduction cascades dedicated to cellular signaling and are a ubiquitous presence in the environment, it is not surprising that microbes have evolved diverse mechanisms to survive the challenges of exposure to antimicrobial drugs.

Drug resistance is of particular importance in fungi as they can cause tremendous economic damage through loss of agricultural yields, as well as life-threatening diseases in both immunocompromised and immunocompetent individuals (49, 123, 129). Invasive fungal infections in patients are notoriously difficult to safely treat with the limited number of drugs that have selective toxicity to fungi, which share close evolutionary relationships with their human hosts (12). Conservative estimates of the cost to the health care system of invasive fungal infections are a staggering $2.6 billion annually in the United States alone, with antifungals comprising the largest proportion of expenditures on anti-infectives in most major medical centers (234). The limited armamentarium of antifungal drugs are often compromised either by severe side effects in the patient, a limited spectrum of antifungal activity, or the emergence of antifungal drug resistance (3, 147).

This review focuses on the evolution of drug resistance in three major human fungal pathogens, Candida albicans, Aspergillus fumigatus, and Cryptococcus neoformans. We begin with a brief introduction to the classes of antifungal drugs currently in clinical use, the clinical realities of antifungal resistance, and the type of stress the antifungals exert on the fungal cell. For each of the pathogens, we then address clinical and experimental findings regarding resistance to these antifungal drugs, with an emphasis on the interplay between signaling molecules, stress responses, and the evolution of drug resistance.

ANTIFUNGAL DRUGS AND CLINICAL ANTFUNGAL RESISTANCE

The arsenal of clinically useful antifungal agents is limited by the number of drug targets in fungi that do not have homologs of similar function and susceptibility to inhibition in humans (209, 224). Most of the antifungal drugs utilized (Fig. 1) target ergosterol, the main sterol of fungal membranes; the biosynthesis of ergosterol; or the biosynthesis of (1,3)-β-D-glucan, a key component of the fungal cell wall (85, 147). The fungicidal polyenes (e.g., amphotericin B) intercalate into ergosterol-containing membranes to form membrane-spanning channels that cause leakage of cellular components and, ultimately, cell death. Polyenes have been clinically exploited for over 50 years, but their utility is often compromised by toxicity to the host, likely due to the similarity between ergosterol and cholesterol in mammalian cell membranes.

The triazoles are the largest class of antifungal drugs in

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clinical use and have been deployed for approximately two decades; they are heterocyclic synthetic compounds that inhibit the fungal cytochrome P450_{14DM} (also known as lanosterol 14α-demethylase) which catalyzes a late step in ergosterol biosynthesis. The drugs bind through a nitrogen group in their five-membered azole ring to the heme group in the target protein and block demethylation of the C-14 of lanosterol, leading to the substitution of methylated sterols in the membrane and resulting in cell membrane stress. Ergosterol biosynthesis occurs in the endoplasmic reticulum. (B) Polyenes bind to ergosterol in the fungal cell membrane, forming membrane-spanning channels that cause leakage of cellular components and osmotic cellular lysis. (C) Flucytosine is deaminated into 5-fluorouracil and, subsequently, 5-fluorodeoxyuridine monophosphate, which inhibits thymidylate synthase and interferes with DNA synthesis; 5-fluorouracil is also converted into 5-fluorouridine triphosphate, which disrupts RNA synthesis. (D) Echinocandins inhibit (1,3)-β-D-glucan synthase (the catalytic subunit is encoded by \textit{FKS1} in \textit{C. albicans}, \textit{C. neoformans}, and \textit{A. fumigatus} and by \textit{FKS1} and \textit{FKS2} in \textit{S. cerevisiae}; \textit{RHO1} encodes a positive regulator of glucan synthase activity), resulting in cell wall stress and a loss of cell wall integrity. +, present; −, absent. (Adapted from reference 32 with permission of the publisher.)
brane, depletion of ergosterol, and accumulation of a toxic sterol intermediate. Triazole antifungal action is generally fungistatic against yeasts, such as *Candida* species, but fungicidal against mold infections, such as *Aspergillus* infections. This delicate clinical point is crucial when treating a patient with profound neutropenia (a lack of an effective number of white blood cells), where the clinician is relying solely on the fungicidal nature of the antifungal for efficacy since the host immune system is absent. Currently there are four triazoles available for clinical use (fluconazole, itraconazole, voriconazole, and posaconazole) and several other agents under investigation in early preclinical and clinical trials. Just as each has unique pharmacokinetic and pharmacodynamic characteristics, it is overly simplistic to infer that antifungal resistance is uniform across the antifungal drugs against a particular fungus.

The echinocandin antifungals are cyclic hexapeptide agents that interfere with cell wall biosynthesis by noncompetitive inhibition of (1,3)-β-D-glucan synthase, an enzyme present in fungi but absent in mammalian cells. This (1,3)-β-D-glucan, an essential cell wall polysaccharide, forms a fibril of three helically entwined linear polysaccharides and provides structural integrity for the fungal cell wall. Disruption of the cell wall causes loss of cell wall integrity and severe cell wall stress (53, 219). In comparison to the activities of the triazoles, the echinocandins are generally fungicidal against yeasts but fungistatic against molds. While the echinocandins have general antifungal activity against *Candida* and *Aspergillus* species, they appear in some clinical studies to be less active against *C. parapsilosis* (138, 153, 180) or *A. terreus* (54), and this class has no clinically relevant antifungal activity against *Cryptococcus neoformans* infection. Currently there are three echinocandins (caspofungin, micafungin, and anidulafungin) available for clinical use.

The patient with an invasive fungal infection is the ultimate standard for investigating antifungal drug resistance. However, large-scale clinical data encompassing all the relevant patient subpopulations are difficult to obtain and interpret. In the absence of such a standard, we are limited to understanding the nuances of common clinical scenarios and employing molecular biological tools to help us explain the present and predict the future. Clinical resistance may be defined as the persistence or progression of an infection despite appropriate antifungal therapy, but antifungal resistance is only one possible etiology leading to ultimate clinical failure. Potential clinical antifungal resistance can often be determined based on epidemiology, without the need for in vitro antifungal susceptibility testing, where certain species are generally more resistant than other species, or through duration of antifungal exposure, much like bacteria. For instance, clinical paradigms dictate that *Candida krusei* and *Candida glabrata* are generally resistant to fluconazole while *Candida lusitaniae* and *A. terreus* are resistant to amphoterin B, and further molecular investigation is not required for the clinician to utilize an appropriate therapy. Understanding the molecular mechanisms that lead to clinical resistance is crucial and in vitro susceptibility testing, coupled with identifying the molecular alterations in the fungus that confer reduced susceptibility to a drug, is paramount to improving clinical response.

**CANDIDA ALBICANS**

As a natural member of the mucosal microbiota, *Candida* species rank as the most common causative agents of invasive fungal infections (129) and cause a broad spectrum of diseases, from thrush and vaginitis in both immunocompetent and immunocompromised individuals to life-threatening systemic disease in immunocompromised patients. Candidiasis, largely caused by *C. albicans*, reigns as the fourth most common cause of hospital-acquired infectious disease (169, 240). The deployment of antifungal drugs has selected both for the emergence of resistance in *C. albicans* and for an increased prevalence of other *Candida* species with different susceptibility profiles, including *C. glabrata, C. parapsilosis, C. tropicalis*, and *C. krusei* (33, 168, 229).

**RESISTANCE TO DRUGS EXERTING CELL MEMBRANE STRESS: THE TRIAZOLES**

Many immunocompromised patients are on long-term prophylaxis with triazoles due to concerns about developing an invasive fungal infection or difficulties in clearing the infection (22), creating favorable conditions for the emergence of resistance. Triazoles inhibit Erg11, blocking the production of ergosterol and resulting in the accumulation of a toxic sterol intermediate that disrupts membrane integrity (Fig. 1), culminating in severe membrane stress (3, 32, 112, 147). *C. albicans* can exploit several cellular stress responses to tolerate exposure to the triazoles and can also acquire high-level resistance by multiple distinct mechanisms (Fig. 2 and Table 1). In both experimental populations and clinical isolates, the emergence of high-level resistance is often accompanied by the acquisition of multiple resistance mechanisms by the same strain (29, 35, 37, 38, 228).

**Alteration of the target enzyme.** One mechanism of triazole resistance that operates by minimizing the impact of the drug on the cell in both laboratory and clinical samples is alteration of the target enzyme, Erg11 (Fig. 2A). At least 12 mutations in Erg11 have been associated with triazole resistance, the majority of which compromise binding of the triazoles to the target (98, 121, 190). Upregulation of *ERG11* has also been associated with triazole resistance (61). An intriguing mechanism involving genomic alterations that amplify the copy number of *ERG11*, as well as another resistance determinant, was recently identified in triazole-resistant strains (198, 199). These alterations include mitotic recombination, gene conversion, and the formation of an isochromosome that confer resistance by increasing the gene dosage of *ERG11* and the gene encoding the transcription factor Taf1 that regulates the expression of multidrug transporters (29, 198, 199).

**Upregulation of multidrug transporters.** Upregulation of the expression of either of two classes of multidrug transporters which efflux triazoles, as well as many other compounds, out of the cell constitutes the second mechanism by which the impact of the drug can be minimized (Fig. 2B). Constitutive upregulation of Mdr1, a member of the major facilitator superfamily of transporters, confers resistance to fluconazole (67, 163, 230). Multiple *cis*-regulatory sequences governing the upregulation of Mdr1 have recently been identified (75, 182, 188), as have key residues in the transporter required for drug efflux...
Recent work has also revealed the identity of the key transcription factor regulating MDR1, Mrr1, and hyperactivating mutations causing constitutive MDR1 overexpression (139).

Constitutive upregulation of the members of the ATP-binding cassette (ABC) transporter superfamily Cdr1 and Cdr2 is the most-frequently encountered triazole resistance mechanism in clinical isolates (67, 163, 230). Multiple cis-regulatory elements have been identified (51), as has the key transcription factor regulating the expression of these transporters, Tac1. (Adapted from reference 32 with permission of the publisher.)

TABLE 1. Antifungal drug resistance mechanisms of C. albicans, C. neoformans, and A. fumigatus

| Antifungal drug class | Candida albicans Description | Cryptococcus neoformans Description | Aspergillus fumigatus Description |
|-----------------------|-----------------------------|----------------------------------|----------------------------------|
| Polyene               | Reduced-ergosterol-content; related species with reduced intrinsic susceptibility, C. lusitaniae | Defective sterol delta8-7 isomerase activity | Related species with reduced intrinsic susceptibility, A. terreus |
| Triazole              | Mutation or overexpression of target P450<sub>ADM</sub> (Erg11), constitutive upregulation of multidrug transporters, loss of function of Erg3, Hsp90- and calcineurin-mediated stress responses; related species with reduced intrinsic susceptibilities, C. krusei and C. glabrata | Mutation or overexpression of target P450<sub>ADM</sub> (Erg11), constitutive upregulation of multidrug transporters | Mutation or overexpression of target P450<sub>DM</sub> (Cyp51), constitutive upregulation of multidrug transporters, reduced drug penetration |
| Echinocandin          | Mutation of target Fks1, compensatory synthesis of cell wall components, PKC cell wall integrity signaling; related species with reduced intrinsic susceptibilities, C. guillermondii and C. parapsilosis | Intrinsic clinical resistance | Mutation of target Fks1, compensatory synthesis of cell wall components, calcineurin-mediated stress responses |
expression in response to drug exposure; however, gain-of-function mutations or increased dosage of the hyperactive alleles, such as by the genomic alterations described for ERG11 above, can confer resistance (29, 198, 199). The emerging theme with multidrug transporters is that altered signaling confers resistance such that mutations in the transcription factors regulating the pumps convert inducible expression upon drug exposure to constitutive high-level expression.

** Cellular stress responses.** In addition to the two mechanisms described above which minimize the impact of the drug on the cell, other mechanisms allow the cell to cope with drug-induced stress. One well-characterized mechanism that falls into this category involves loss of function of Erg3 in the ergosterol biosynthetic pathway (Fig. 2C), which blocks the accumulation of toxic sterol intermediates when Erg11 is inhibited by the azoles (3, 32). While this mechanism of resistance has been identified in clinical isolates (87), the clinical relevance is less clear (135). Some resistance mechanisms may span both categories, with dual roles in minimizing the impact of the drug on the cell and enabling the cell to cope with stress. For example, the transcription factor Mr1 regulates targets in addition to MDR1, including oxidoreductases, which may help prevent drug-induced cell damage resulting from the generation of toxic molecules (139). The transcription factor Tac1 also regulates targets in addition to CDR1 and CDR2, including a putative glutathione peroxidase, sphingosine kinase, and phospholipid flippase, suggesting roles in oxidative stress response and lipid metabolism (111). In all conditions when the triazoles do effectively inhibit their target, cellular stress responses are required for survival; this applies both to the basal tolerance of wild-type cells and the resistance phenotypes of specific mutants.

As a key regulator of cell signaling in all eukaryotes, the Ca2+-calmodulin-activated protein phosphatase calcineurin provides a poignant example of the role of signaling molecules in mediating crucial responses to antifungal drugs (Fig. 2D) (20, 66, 206). Calcineurin is dispensable for growth in C. albicans under standard laboratory conditions but mediates essential responses to diverse stresses, including exposure to serum (18, 21) and alkaline pH (94), as well as agents inducing cellular stress responses (39, 150, 191). The fungal metabolites and immunosuppressants cyclosporine and FK506 bind to immunophilins, forming drug-protein complexes that are potent inhibitors of calcineurin function (109) and are synergistic with triazoles, as well as other agents mediating membrane stress responses (118, 120, 150). While cyclosporine and FK506 can also inhibit multidrug transporters (50, 79, 179, 196), the specificity of their effects on calcineurin in mediating crucial responses to triazoles has been validated in C. albicans. The FK506 binding protein FKBP12, which FK506 must bind to in order to inhibit calcineurin, is required for FK506 synergy with triazoles (39), and the synergy persists even in strains lacking the multidrug transporters associated withazole resistance (119). Furthermore, abrogating calcineurin function genetically by deleting the regulatory subunit encoded by Cnb1 or the catalytic subunit encoded by Cna1 renders cells hypersensitive to azoles (39, 191). In addition to mediating the azole tolerance of wild-type cells, calcineurin is also required for azole resistance mediated by loss of function of Erg5, as well as for resistance due to multiple mechanisms found in clinical isolates (34, 36).

One downstream effector of calcineurin, the transcription factor Crz1, has been implicated inazole resistance in C. albicans (Fig. 2D). Crz1 is dephosphorylated by calcineurin, upon which it translocates to the nucleus, activating the expression of a suite of genes involved in signaling pathways, ion/small-molecule transport, cell wall integrity, and vesicular trafficking (84). CRZ1 deletion strains have moderate triazole sensitivity, which can be further exacerbated by the addition of a calcineurin inhibitor, suggesting that other calcineurin-dependent mediators of triazole resistance remain to be identified (84, 151, 193). In *Saccharomyces cerevisiae*, the resident proteins Hph1 and Hph2 of the endoplasmic reticulum are downstream effectors of calcineurin that mediate the response to triazoles; however, no *C. albicans* homologs of these gene products have yet been identified (34).

A global regulator of cellular signaling and stress responses that plays a key role in triazole resistance, in large part via calcineurin, is the molecular chaperone Hsp90 (Fig. 2D). Hsp90 is essential in all eukaryotes and regulates the form and function of a diverse set of client proteins, many of which are key regulators of cellular signaling (162, 174, 177, 239, 242). Hsp90 interacts with the catalytic subunit of calcineurin and is required for its function (78, 95). Pharmacological inhibition of Hsp90 with the microbial metabolites geldanamycin and radicicol, which bind with high affinity to Hsp90’s unusual ATP binding pocket to block ATP-dependent chaperone activity (187, 231), prevents the rapid emergence of drug resistance in *C. albicans* in a laboratory setting (36). Hsp90 inhibitors also reduce the resistance of Erg3 loss-of-function mutants and clinical isolates that have acquired resistance by multiple mechanisms (34, 36). Genetic studies validated the key role of Hsp90 in the evolution and maintenance of fluconazole resistance in *S. cerevisiae* (36) and in *C. albicans* (L. E. Cowen, unpublished data). In all cases tested, similar results were observed with the newer-generation triazole voriconazole (36).

In all *S. cerevisiae* and *C. albicans* mutants tested, inhibition of calcineurin function phenocopies inhibition of Hsp90 function, consistent with calcineurin being a key mediator of Hsp90-dependent azole resistance.

The interconnected stress responses mediating triazole resistance also include the evolutionarily conserved cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway (9, 113, 156, 184). The production of the central messenger of this pathway, cAMP, is regulated by adenylate cyclase and cyclase-associated proteins. The deletion of these proteins in *C. albicans* or *S. cerevisiae* confers hypersensitivity to triazoles that is partially reversed by the addition of cAMP to the medium (80). Notably, in *S. cerevisiae*, PKA phosphorylates and negatively regulates Crz1 activity by inhibiting its nuclear import (83). Given that PKA negatively regulates Crz1, one might expect the deletion of PKA to enhance triazole resistance via Crz1 activity rather than reduce it; thus, the specific effects of PKA on triazole resistance may be distinct from calcineurin and remain to be elucidated. RNA analysis suggests that a defect in triazole-dependent upregulation of the multidrug transporter
to amphotericin B has been reported in clinical isolates of rather than drug resistance (62). The emergence of resistance with polyenes have been poor aqueous solubility and toxicity isolates is associated with reduced ergosterol content in the cell patients treated with amphotericin B (210). Resistance of clinical resistance.

Another interconnected signaling molecule that plays a role in triazole resistance is protein kinase CK2 (23). CK2 and its homologue CKA1 encode catalytic subunits of protein kinase CK2. An insertional mutagenesis screen revealed that mutations in cka2 confer fluconazole resistance and that this resistance is suppressed by CKA1 overexpression. cka2Δ mutants overexpress the efflux transporters Cdr1 and Cdr2, but this only partially explains their resistance phenotypes. The calcineurin inhibitor cyclosporine abrogates cka2-mediated resistance, as does loss of function of Crz1. Expression analyses argue that Cka2 and Crz1 act through distinct mechanisms, given that Crz1 was not required to mediate the gene expression changes observed in the cka2 deletion mutant. Further supporting the key role of signaling molecules in mediating responses to triazoles, mutants lacking either of the two-component signal transduction proteins Ssk1 or Chk1 are hypersensitive to both fluconazole and voriconazole (27).

An intriguing way in which signaling pathways can mediate triazole resistance that has taken center stage of late is the formation of fungal biofilms. Biofilms represent a complex architecture of different cell types that form when freely moving (planktonic) cells interact with particular surfaces, such as plastics and intravenous catheters (19, 52, 142, 145). While this may be distinct from the stress responses discussed above, it highlights the importance of cellular signaling in drug resistance phenotypes. The formation of a biofilm involves a fundamental remodeling of the cellular state, with alterations of the transcriptional program, the induction of morphological changes, the production of an extracellular matrix, and changes in cell-to-cell communication. C. albicans biofilms are resistant to many antifungal drugs, including triazoles both in vitro and in vivo (19, 52, 142), and are a difficult clinical treatment situation, generally necessitating the removal of the patient’s catheter in order to effect a complete cure. The mechanisms of triazole resistance of C. albicans biofilms are likely to be multifactorial, involving upregulation of multidrug transporters, reduced drug diffusion, and reduced growth rate, as well as alterations of the plasma membrane and cell wall (52, 143). It is clear that multiple signaling pathways are required to sense environmental cues and trigger that cascade of cellular events that ultimately manifests in biofilm-mediated triazole resistance.

RESISTANCE TO DRUGS EXERTING CELL MEMBRANE STRESS: THE POLYENES

In stark contrast to the azoles, the major clinical problems with polyenes have been poor aqueous solubility and toxicity rather than drug resistance (62). The emergence of resistance to amphotericin B has been reported in clinical isolates of C. albicans, as well as other Candida species recovered from patients treated with amphotericin B (210). Resistance of clinical isolates is associated with reduced ergosterol content in the cell membrane, in some cases attributed to defective Erg3 function (87, 146). A laboratory study involving directed mutations of genes in the ergosterol biosynthetic pathway found that erg3 homozygous deletion mutants are hypersensitive to amphotericin B while erg11 homozygous deletion mutants, selected by plating erg11 heterozygous deletion mutants on polylene-containing plates, are resistant (192).

Compared to the azoles, there is little known about links between amphotericin B resistance and cellular signaling. This may be due to the fact that amphotericin B is fungicidal against Candida species and causes leakage of cytoplasmic contents, while the azoles are fungistatic, causing growth arrest and the induction of membrane stress responses. That said, the exposure of wild-type cells to amphotericin B at a concentration that inhibits growth by 50% does modulate the expression of several genes involved in small-molecule transport and cellular stress response (110). The signaling pathways involved in the elaboration of biofilms provide one commonality between resistance to azoles and to amphotericin B, as C. albicans biofilms show robust resistance to both. Neither Hsp90 nor calcineurin has been directly implicated in mediating resistance to amphotericin B as of yet. However, a randomized, blinded clinical trial with a human recombinant monoclonal antibody against Hsp90 in conjunction with amphotericin B therapy found significant clinical improvement over amphotericin B alone (126, 154). It is unlikely that the antibody could enter an intact fungal cell to inhibit cellular signaling mediated by the cytosolic chaperone Hsp90; the mechanism by which Mycograb exerts its antifungal activities remains enigmatic but may involve effects on Hsp90 at the cell surface.

RESISTANCE TO DRUGS EXERTING CELL WALL STRESS

The target of the echinocandin antifungals, (1,3)-β-D-glucan synthase, is a multisubunit enzyme complex composed of at least two subunits, Fks1 and Rho1 (Fig. 1). Fks1 is thought to be the catalytic subunit (194) and the target of the echinocandins (58, 59). Rho1, a GTP-binding protein in the Rho/Rac subfamily of Ras-like GTPases, is a positive regulator of glucan synthase activity (178). The mechanistic nature of the interaction between echinocandins and glucan synthase remains enigmatic. Clinical resistance to echinocandins is still rare but has been reported in patients undergoing treatment with these agents (103, 134, 158).

Alteration of the target enzyme. As with the azoles, one resistance mechanism by which cells can minimize the impact of the drug is mutation of the drug target (Fig. 3A). The most common mechanism of resistance to echinocandins observed to date is mutation in Fks1, most frequently within two highly conserved regions (165). Mutations in Fks1 conferring echinocandin resistance have been identified in laboratory studies with S. cerevisiae and C. albicans (11, 60, 96), as well as in clinical isolates of C. albicans (103, 134, 158). Fks1 mutations characteristically decrease the echinocandin sensitivity of glucan synthase such that the mutant enzyme may be ~1,000-fold less sensitive to the drug (165). Naturally occurring polymorphisms in Fks1 may account for the reduced echinocandin susceptibility of several non-albicans Candida spp., including C. parapsilosis and C. guilliermondii.

Upregulation of multidrug transporters. In contrast to the triazoles, multidrug efflux transporters do not seem to provide
an important mechanism for minimizing the impact of the echinocandins on the cell. Overexpression of the ABC transporter Cdr2 confers only a very moderate increase in caspofungin resistance in agar plate assays (197). A comprehensive study in which Cdr1, Cdr2, or Mdr1 was overexpressed in either C. albicans or S. cerevisiae revealed only subtle changes in responses to echinocandins, which were evident on solid substrate but not in liquid medium (144). Larger-scale studies found potent in vitro activity of echinocandins against hundreds of triazole-resistant C. albicans strains overexpressing multidrug transporters, suggesting that this is not a clinically relevant mechanism of resistance (7, 167, 170, 171).

**Cellular stress responses.** As with the triazoles, there is an intimate connection between signaling pathways mediating cellular stress responses and the ability to survive the stress exerted by the echinocandins (Fig. 3B). By inhibiting the synthesis of (1,3)-β-D-glucan, the echinocandins cause loss of cell wall integrity and induce an acute cell wall stress (165). Rho1, which positively regulates the activity of (1,3)-β-D-glucan synthase, also orchestrates the protein kinase C (PKC) cell wall integrity signaling pathway that is responsible for remodeling the cell wall periodically through the cell cycle and in response to various stresses (104, 106). While less is known about key mediators of cellular responses to echinocandins in C. albicans, in S. cerevisiae the expression of genes involved in cell wall integrity and cell wall biosynthesis is modulated in response to exposure to echinocandins and plays a crucial role in tolerating echinocandin-induced stress (181). Genome-wide screens of S. cerevisiae also suggest a role for the PKC cell wall integrity pathway, as well as compensatory synthesis of other cell wall components chitin and mannan in tolerance to echinocandins (105, 122). The results of recent work suggest that the PKC cell wall integrity pathway may also contribute to a “paradoxical effect” where the activity of echinocandins against C. albicans is attenuated at higher concentrations (232). A C. albicans mitogen-activated protein kinase (MAPK) in the PKC pathway, Mkc1, is overexpressed at the elevated echinocandin concentrations, and mutants that are null for mkc1 no longer demonstrate this effect (233). Chitin levels are also increased in C. albicans at the elevated echinocandin concentrations, though these increases may also occur at lower levels where echinocandin activity is not attenuated (166, 212).

There are several links that suggest that calcineurin signaling might be intimately connected with cellular responses to the echinocandins, though this is much less clear than with the triazoles. The results of recent studies have demonstrated that the regulation of both chitin synthase gene expression and chitin synthesis is coordinated by the PKC, high-osmolarity glycerol response MAPK, and calcineurin signaling pathways in C. albicans (140). The calcineurin-dependent transcription factor Crz1 is required for activation of the expression of several of the chitin synthases in response to stress conditions (140). Given that the upregulation of cell wall components may provide protection against echinocandin-induced cell wall stress, this suggests a possible link between calcineurin and cellular responses to echinocandins.

In connection with the “paradoxical effect” discussed above, the calcineurin inhibitor cyclosporine abrogates the enhanced growth of C. albicans at elevated echinocandin concentrations (233). While this may suggest a role for calcineurin in mediating a protective response, the possibility that the effects of cyclosporine were mediated through targets other than calcineurin has yet to be ruled out. Deletion of the regulatory subunit of calcineurin required for its activation, Cnb1, had no effect on the echinocandin resistance of C. albicans in one study (39), while in another study, deletion of the catalytic subunit of calcineurin, Cna1, enhanced the killing activity of caspofungin (191). Notably, FKS1 is essential in C. albicans

**FIG. 3.** C. albicans echinocandin resistance mechanisms. (A) Mutation of (1, 3)-β-D-glucan synthase, the target of the echinocandins encoded by FKS1, confers resistance to echinocandins by minimizing the impact of the drug on the cell. (B) Rho1 is a positive regulator of glucan synthase, and it contributes to tolerance of the echinocandins by mediating stress responses, including activation of the PKC cell wall integrity pathway and upregulation of synthesis of other cell wall components, such as chitin. (Adapted from reference 32 with permission of the publisher.)
(58); however, the essential (1,3)-β-D-glucan synthase activity
is encoded by two partially redundant genes, FKS1 and FKS2, in S. cerevisiae (128, 241). The expression of FKS2 is regulated by PKC signaling and by calcineurin via Cez1 (128, 202, 241). Thus, S. cerevisiae fks1Δ mutants are hypersensitive to the calcineurin inhibitors cyclosporine and FK506 (59, 63) and are synthetic lethal with deletion of cnb1, due to a loss of FKS2 expression (63, 105, 241). The role of calcineurin in mediating cellular responses to echinocandins in C. albicans warrants further investigation.

If calcineurin signaling does play a substantial role in responses to echinocandins, then one might also predict a role for the molecular chaperone Hsp90. Notably, the functional dependence of calcineurin on Hsp90 has been established for S. cerevisiae and for the protozoan parasite Plasmodium falciparum (78, 95) but has not yet been explored in other fungal species. Hsp90 has yet to be implicated in mediating resistance or tolerance to echinocandins, though only a small number of strains have been examined to date (36).

Unlike with the triazoles, the alterations in cellular signaling mediating the elaboration of C. albicans biofilms do not seem to confer resistance to the echinocandins (52) but may have other intriguing connections with enhancing the production of (1,3)-β-D-glucan. Increased (1,3)-β-D-glucan content is found in C. albicans cell walls from biofilms compared to the (1,3)-β-D-glucan content in planktonic cells, and this is associated with increased binding of fluconazole (143). Concentrations of (1,3)-β glucanase that have no impact on planktonic cells result in dose-dependent killing of biofilm cells, and (1,3)-β glucanase can enhance the activities of fluconazole and amphotericin B against biofilms.

**ASPERGILLUS FUMIGATUS**

No other invasive fungal pathogen has prompted such lengthy debate and discussion over optimal antifungal therapy, both in the literature and at the bedside, as invasive aspergillosis. This has forced clinicians and scientists alike to explore potential resistance mechanisms, but ultimate clinical relevance might instead be related to patient factors (i.e., recovery of neutropenia or cessation of glucocorticoid therapy) and not intrinsically related to the susceptibility of the fungus itself. An international surveillance project examined Aspergillus isolates from 2003 (133), and compared to Aspergillus isolates studied in 2000 (173), found that recent A. fumigatus isolates were generally two- to fourfold less susceptible to amphotericin B, twofold less susceptible to voriconazole and ravuconazole, and remained as susceptible to itraconazole. A multicenter examination of 697 Aspergillus sp. strains over five years (42) revealed A. nidulans with the highest MIC of (>8 µg/ml) against itraconazole, voriconazole, and posaconazole and amphotericin B the least active, suggesting that A. nidulans strains were generally less susceptible than A. fumigatus. Later testing of 446 Aspergillus clinical and environmental strains revealed a native reduced susceptibility of A. terreus and A. flavus to amphotericin B and strains of A. niger and A. nidulans with generally higher MICs to itraconazole (4), but no resistance detected in 171 strains of A. fumigatus. While there are only limited large-scale epidemiologic studies demonstrating resistance of Aspergillus to antifungal agents, the molecular exploration of antifungal resistance has revealed numerous pivotal findings (Table 1).

**RESISTANCE TO DRUGS EXERTING CELL MEMBRANE STRESS: THE TRIAZOLES**

Itraconazole resistance in A. fumigatus was only first described in 1997, from two patients with invasive aspergillosis who failed itraconazole therapy despite good serum levels, and the isolates had itraconazole MICs of >16 µg/ml (56). This in vitro resistance was then confirmed in a murine model; however, mice infected with the same resistant strain but treated with a higher dose of itraconazole demonstrated improved survival (P = 0.005). Sterol analysis of the resistant strains showed that one strain possessed a reduced ergosterol content and greater quantities of sterol intermediates, while another resistant strain displayed slightly higher ergosterol content and lower intermediate sterol concentrations. Additionally, one resistant isolate had a low intracellular concentration of itraconazole, whereas the other two isolates required much more itraconazole to inhibit the ergosterol pathway. Further murine model work using itraconazole-susceptible and itraconazole-resistant isolates showed in vivo correlation with the in vitro susceptibility patterns (44, 55). Another study examined four itraconazole-resistant clinical isolates by mass spectrometry and found that the sterol compositions were similar in the resistant and susceptible strains, suggesting that resistance was not related to alterations in the ergosterol synthesis pathway (46). Collectively, these findings suggest that several molecular mechanisms of itraconazole resistance might be operating in A. fumigatus, including reduced intracellular itraconazole concentration due to an efflux pump, reduced penetration of the drug, and modification or overexpression of the target enzyme.

**Alteration of the target enzyme.** Triazoles selectively inhibit the cytochrome P450-dependent C-14 lanosterol α-demethylase, a key enzyme in ergosterol biosynthesis, and similar to the work on Candida species, this enzyme has been a focus of the exploration of molecular antifungal resistance in Aspergillus species. Using A. nidulans isolates (152), laboratory-derived itraconazole-resistant isolates were found to have acquired resistance by extra copies of pdmA, the gene for A. nidulans P450 C-14 lanosterol α-demethylase; the extra copies increased the MIC of itraconazole 36-fold over the MIC of wild-type controls. The transformation of A. fumigatus isolates with this resistance plasmid led to a fourfold increase in itraconazole resistance but did not alter amphotericin B sensitivity, demonstrating that triazole resistance might be related to overexpression of the drug target.

There are two distinct but related cytochrome P450-dependent C-14 lanosterol α-demethylase proteins (Cyp51 proteins) in A. fumigatus, encoded by cyp51A and cyp51B (130), and most antifungal resistance work on A. fumigatus has focused on point mutations in these genes. In one study of spontaneous posaconazole-resistant mutants (117), the gene expression of cyp51A, cyp51B, mdr1, and mdr2 without antifungal exposure showed no change compared to that of posaconazole-sensitive strains, and the uptake of [14C]posaconazole was also unchanged. Each resistant strain had a single nucleotide substitution at codon 54 in cyp51A; in the four isolates with moderate resistance, the substitution was glycine changed to glutamate.
or arginine, but in the mutant with high-level resistance, the glycine was mutated to tryptophan. As expected from the structural similarity of posaconazole to itraconazole, the posaconazole-resistant strains exhibited resistance to itraconazole, but not voriconazole, a finding that differs from results for C. albicans, where fluconazole-resistant strains are frequently cross-resistant to itraconazole and voriconazole (40).

Numerous additional mutations in the target of the triazoles have been associated with antifungal resistance in A. fumigatus. In one study, sequences of the cyp51A and cyp51B genes revealed a single base change at codon 54 mutating glycine in six resistant strains, while there were no point mutations in any itraconazole-sensitive strains. Additionally, five other strains showed a mutation at codon 236 to valine, lysine, or threonine. Most mutations were in cyp51A, and while there were five substitutions in cyp51B, only two resulted in an amino acid substitution, at codon 42 and at codon 387, highlighting the heterogeneity of resistance (57). In another study, five out of eight strains had a single substitution in codon 220 where methionine was mutated to valine, lysine, or threonine. None of those five strains had concurrent mutations at codon 54, but they did have other mutations in Cyp51A (131). A mutation at codon 98 led to resistance only in combination with a tandem duplication of a 34-bp sequence of the cyp51A promoter, finding that differs from results for C. albicans, where fluconazole-resistant strains are frequently cross-resistant to itraconazole and voriconazole (40).

Inducible triazole resistance was also shown when the growth of a single strain in the presence of a subinhibitory level of itraconazole led to 10 mutants with various levels of drug resistance and growth rates, as well as variation in the percent inhibition of ergosterol biosynthesis. Sequence analysis revealed different mutations in both cyp51A and cyp51B. Most mutants showed constitutive overexpression of mdr3 and mdr4, and some showed overexpression of atrF. In the presence of itraconazole, gene expression increased in most resistant strains, but not all, again suggesting multiple mechanisms (47).

Acquired itraconazole resistance was first reported in 1999 in a patient with invasive aspergillosis treated with itraconazole for 5 months (43). Two isolates recovered before the commencement of antifungal treatment had MICs of 0.5 μg/ml, while two isolates obtained after therapy possessed elevated MICs of >16 μg/ml, with confirmed resistance in a murine model. One later study cloned the ATP-binding cassette transporter atrF by using a probe for CDR1 from C. albicans (201). Without the stress of itraconazole, the levels of atrF were barely detectable in sensitive and resistant strains. In the presence of itraconazole, atrF expression increased fivefold in the resistant strain but was unchanged in sensitive strains.

Spontaneous laboratory-selected itraconazole-resistant mutants showed that itraconazole uptake was reduced as intracellular concentrations of [3H]itraconazole were reduced by 80% (116). The later use of a respiratory inhibitor, carbonyl cyanide m-chlorophenyl hydrazone, revealed similar reduced accumulation in resistant and susceptible strains, suggesting that the reduced itraconazole concentrations in the mutant are probably due to a reduced penetration of the drug into cells, as opposed to an energy-dependent process, such as efflux (56).

In another study, laboratory-derived voriconazole-resistant A. fumigatus isolates showed no significant rise in their amphotericin B MICs, and only a 1.5-fold increase in their itraconazole MICs, and only a 1.5-fold increase in their posaconazole MICs. However, all final MICs were still clinically susceptible, and the resistant isolates showed an accumulation of voriconazole similar to that in the control when mycelia were incubated with the drug, suggesting that it is unlikely that resistance is due to decreased accumulation of the drug (115).

**RESISTANCE TO DRUGS EXERTING CELL MEMBRANE STRESS: THE POLYENES**

*A. terreus* has been consistently shown via in vitro antifungal susceptibility testing (213), in vivo animal models (71, 223), and detailed clinical series (100, 203) to be resistant to amphotericin B. In the largest clinical series, therapy with amphotericin B was directly shown to contribute to clinical failure, while therapy with voriconazole led to greater clinical success (203). While there are occasional reports of in vitro sensitivity to amphotericin B, the clinical paradigm has been to avoid therapy with amphotericin B due to concerns about resistance and repeated clinical failure against A. terreus invasive aspergillosis. *A. nidulans* has also been shown to have higher rates of amphotericin B resistance, both in vitro and clinically (4, 90).

These clinical correlations are essential for invasive aspergillosis and antifungal resistance, as the results of in vitro testing can be misleading. One study found no relationship between in vitro amphotericin B susceptibilities and clinical outcome in 18
patients infected with different species of *Aspergillus* (108). Risk factors that were related to overall clinical outcome were all host related, suggesting that in vitro antifungal susceptibility testing provided little additional clinically meaningful information regarding the likelihood that a patient would fail therapy. Other studies have shown a poor in vitro-in vivo correlation in animal models (81, 148). However, some reports have demonstrated a link between in vitro susceptibility to amphotericin B and clinical outcome (101).

In an age of the use of empirical antifungal therapy for a patient with suspected disease, the issue of preexposure to an antifungal as a catalyst for future antifungal resistance is crucial. One study tested isolates from six patients with fatal invasive aspergillosis who were receiving amphotericin B before culture isolation and showed in vitro amphotericin B susceptibilities that were similar to those of isolates from 35 patients with no prior exposure to amphotericin B. These data indicate that the emergence of resistance to amphotericin B is uncommon during therapy for invasive aspergillosis (137). These findings were confirmed in a larger study analyzing 200 sequential *A. fumigatus* isolates from 26 patients which determined that the MICs to amphotericin B (n = 100) or itraconazole (n = 91) were similar both pre- and postexposure, leading to the conclusion that the emergence of resistance while on therapy was infrequent (45).

**RESISTANCE TO DRUGS EXERTING CELL WALL STRESS**

Documented clinical echinocandin resistance is rare (64), so it is unclear if one *Aspergillus* species or another favors resistance (Table 1). In a large clinical trial using the echinocandin micafungin, 0 out of 10 patients infected with *A. terreus* had a complete or partial response, in contrast to a 35.6% (80 out of 225) favorable response in patients infected with all isolates (54). It is clear that the echinocandins have been clinically useful against clinical triazole resistance. Micafungin was active both in vitro and in vivo against itraconazole-resistant *A. fumigatus* and *A. terreus* strains with decreased sensitivity to amphotericin B (225), while one patient with clinical itraconazole failure and documented in vitro resistance was cured with caspofungin (160).

**Alteration of the target enzyme.** In one study, two classes of *A. fumigatus* mutants were isolated that exhibited reduced susceptibility to caspofungin (70). A site-directed mutation in fks1, encoding the catalytic subunit of glucan synthase, conferred a 16-fold reduction in susceptibility. A second class of spontaneous mutants displayed an inducible resistance at a higher level of antifungal concentration but contained neither an fks1 point mutation nor changes in target gene expression. In another study, a point mutation of serine to proline in fks1 codon 678 was engineered in *A. fumigatus* (185) because an equivalent mutation in Fks1 was known to confer echinocandin resistance in *Candida* isolates (11). The mutation conferred resistance to all three echinocandins and led to an increase of 20- to 120-fold of 50% inhibitory concentration values, which is characteristic of drug-resistant phenotypes.

Another study demonstrated that the antifungal efficacies of all three echinocandin antifungals were greatly reduced in the presence of 50% human serum (155). Since the echinocandins are extensively bound to serum proteins, the direct effects of serum inhibition on *Aspergillus* glucan synthase were tested: increasing amounts of serum (0 to 50%) increased the 50% inhibitory concentration of the enzyme, suggesting that serum exerts its effect through a direct drug interaction.

**Cellular stress responses.** The search for optimal antifungal therapy for primary disease or resistant strains through novel mechanistic approaches has prompted investigations into cellular signaling (41, 69). While studies have explored cell signaling related to pathogenicity (107, 127, 149) and nonantifungal stresses (220, 243) in *A. fumigatus*, there has been little work directly pertaining to antifungal resistance.

The calcineurin pathway is implicated in several major human pathogenic fungi (206), and investigations of *A. fumigatus* have not specifically focused on resistance mechanisms but instead on the manipulation of cell signaling pathways to augment conventional antifungal therapy. An initial in vitro study evaluated combinations of caspofungin or itraconazole with calcineurin inhibitors and found that although calcineurin inhibitors showed little intrinsic antifungal activity, combinations of these inhibitors with caspofungin or itraconazole produced a positive interaction (91). Extensive studies using multiple testing formats revealed that the combination of caspofungin with calcineurin inhibitors demonstrated a consistently positive interaction, including converting the normally fungistatic caspofungin to a fungicide (207). Specifically, antifungal synergy was seen with calcineurin inhibitors paired with cell wall-active echinocandins and no increased activity was seen with cell membrane-active triazoles or polyenes. Clinical isolates from transplant patients with long-term exposure to calcineurin inhibitors had in vitro susceptibility patterns similar to those of isolates from oncology patients not receiving calcineurin inhibitors (208), suggesting that long-term exposure to these drugs in vivo does not decrease antifungal activity or select for drug resistance.

A mutant of *A. fumigatus* lacking the calcineurin A (*cnaA*) catalytic subunit exhibited defective hyphal morphology related to apical extension and polarized growth, drastically decreased filamentation, and a profound attenuation of pathogenicity (204). Calcineurin inhibition, via pharmacologic inhibitors or the *cnaA* mutant, led to morphological hyphal defects similar or superior to those found with echinocandin treatment of *A. fumigatus*. This hyphal dysmorphic effect is even more significant with the *cnaA* mutant than with FK506 treatment, suggesting that current calcineurin inhibitors are not completely effective at blocking the calcineurin pathway’s impact on hyphal growth and therefore allowing the possibility that improved drugs for hyphal growth inhibition by blocking calcineurin signaling could be designed. Supporting the hypothesis that calcineurin inhibition operates through cell wall biosynthesis, (1,3)-β-D-glucan measurements decreased following calcineurin inhibition and antifungal synergy was seen with calcineurin inhibition combined with either cell wall inhibition or chitin inhibition (205).

Glycophosphatidylinositol (GPI)-modified proteins play an important role in fungal cell wall organization (102). The ECM33/SPS2 family proteins have features typical of GPI-anchored proteins. The deletion of *S. cerevisiae* ECM33 resulted in a weakened and disorganized cell wall (157), and the *C. albicans* Ecm33 is required for normal cell wall architecture.
Disruption of the *A. fumigatus ecm33* resulted in resistance to caspofungin and Congo red, as well as increased virulence in an immunocompromised mouse model (189); the mutant showed no increased resistance to the cell membrane-active antifungals itraconazole and amphoteracin B. The increased resistance to the cell wall-active agents stands in contrast to the findings of increased sensitivity to Congo red and Calcofluor white in both *S. cerevisiae* and *C. albicans ecm33* mutants (125, 157). It was postulated that the disruption of *ecm33* leading to caspofungin resistance might be due to a possible increase in chitin and (1,3)-β-glucan in the cell wall, thereby compensating for the reduced synthesis of (1,3)-β-glucan (189).

**CRYPTOCOCCUS NEOFORMANS**

*C. neoformans* is a common cause of meningoencephalitis in immunocompromised individuals, with mortality rates of 10 to 15% even in developed countries (28). The incidence of this infection increased dramatically during the height of the AIDS pandemic, and it remains a major opportunistic infection in areas of the world where AIDS is not successfully managed. Even in the era of highly active antiretroviral therapy, cryptococcosis remains a common infection among the expanding immunocompromised population that has accompanied the aggressive use of immunosuppressants for cancer and organ transplantation. In addition to the inherent challenges of treating invasive fungal infections, the treatment of cryptococcal meningitis requires therapeutic agents with sufficient penetration into the central nervous system. Treatment of cryptococcal disease has relied on triazoles, amphotericin B, and flucytosine. For all of these agents, increased in vitro drug resistance is a concern (125). Disruption of the *A. fumigatus ecm33* has largely been documented through clinical experience with AIDS patients undergoing fluconazole therapy (5, 15, 17, 161, 221).

As with *C. albicans* and *A. fumigatus*, triazole resistance in *C. neoformans* has been associated with alterations in the *P450 lanosterol 14α-demethylase* target enzyme encoded by *ERG11* (Table 1). For example, the fifth isolate from a series of five sequential isolates recovered from an AIDS patient with recurrent meningitis demonstrated fluconazole resistance and a G484S substitution in Erg11 relative to the fluconazole-sensitive isolates that were recovered earlier from the same patient (186). A study that examined 11 clinical isolates found that three isolates with low-level resistance had changes in the affinity of the target enzyme for fluconazole, while the four isolates with high-level resistance had decreased cellular content of fluconazole (221).

A mechanism of resistance that is consistent with the reduced intracellular accumulation of triazoles observed in the four clinical isolates with high-level resistance is the upregulation of multidrug transporters, as is common in triazole-resistant *C. albicans* isolates. *CnMDR1* is a gene encoding a protein related to several eukaryotic multidrug resistance proteins that has been identified, cloned, and characterized from a clinical isolate of *C. neoformans*, but it is unknown if it has a role in antifungal drug resistance (216). One study obtained a fluconazole-resistant mutant from an initially sensitive clinical isolate by in vitro exposure to the antifungal and subsequently identified a DNA overexpressed in the resistant mutant that encoded an ABC transporter termed *CnAFR1* (176). *CnAFR1* was confirmed to confer resistance, as disruption of the gene conferred enhanced susceptibility to fluconazole while reintroduction restored resistance.

An intriguing pattern of cellular response to triazoles termed heteroresistance has been reported for *C. neoformans*, though the molecular mechanisms involved remain enigmatic. Heteroresistance has been observed among clinical isolates such that a single cell gives rise to progeny with a heterogeneous composition of resistance phenotypes with mostly susceptible cells but some that are highly resistant to fluconazole or voriconazole, at frequencies ranging from 7 × 10⁻³ to 4.6 × 10⁻² (136). Clones producing homogeneous resistant cells can be selected by exposure to high antifungal concentration; however, the original heteroresistance phenotype is restored upon passage in the absence of antifungal. This resistance is unrelated to prior drug exposure and is not affected by pH or osmolarity of the medium. Strikingly, the resistance is affected by temperature, as it is suppressed at 35°C and abolished at 40°C. A subsequent study examining 107 clinical isolates found fluconazole heteroresistance in 5 isolates and confirmed both that fluconazole resistance could be developed by selection of heteroresistant clones in the presence of fluconazole and that heteroresistance was suppressed at increased temperatures (236). Notably, separate from the phenomenon of heteroresistance, the patterns of mutation to fluconazole resistance in *C. neoformans* are complex. One study examining 21 isolates for the frequency of mutants able to grow on medium with 8 μg/ml fluconazole found that there was no significant correlation between the MIC of the parental strain and the frequency of resistant mutants produced, nor was there a correlation between the parental MIC and the resulting MIC of the selected
mutants, suggesting a dynamic and heterogeneous mutation process (235).

The heterogeneous mutation to triazole resistance and the heteroresistance phenotypes that are contingent on temperature raise the intriguing possibility that cellular stress responses may play a role in triazole resistance in C. neoformans. One study explored the synergistic potential of combining a calcineurin inhibitor with azoles (48). The authors found that the calcineurin inhibitor FK506 had synergistic activity with fluconazole; combined use of these agents resulted in an ~30-fold decrease in the MIC of FK506 and a 4-fold decrease in the MIC of fluconazole. The synergistic activity of FK506 with fluconazole did not depend on the presence of calcineurin or FKBP12, suggesting that FK506 enhances fluconazole action against C. neoformans by a calcineurin-independent mechanism, perhaps through the inhibition of multidrug transporters.

The other class of antifungal drug that remains an important component of the therapeutic strategy for cryptococcal infections and targets the cell membrane is the polyenes, principally amphotericin B. Resistance to amphotericin B remains rare, at <1% of clinical isolates, based on a global survey of the antifungal susceptibilities of 1,811 clinical C. neoformans isolates (172). However, resistance has been documented both in clinical isolates and in response to in vitro selection. An amphotericin B-resistant isolate from an AIDS patient was found to have defective sterol Δ8-7 isomerase activity (88). Isolates selected in vitro that were cross-resistant to both amphotericin B and fluconazole showed reduced cellular accumulation of the antifungals, suggesting a possible role for multidrug transporters (82). Another means by which C. neoformans can acquire resistance to amphotericin B is through the formation of biofilms, which are more resistant both to triazoles and to this polyene than planktonic cells (124).

RESISTANCE TO DRUGS EXERTING CELL WALL STRESS

C. neoformans is resistant to the echinocandins both in vitro and in vivo (1, 13, 93). This intrinsic resistance is surprising given that the C. neoformans gene encoding the (1,3)-β-glucan synthase Fks subunit is essential for growth and that (1,3)-β-glucans are found in the cell wall (215). One possible explanation for the resistance of C. neoformans to the echinocandins is that the target, (1,3)-β-glucan synthase enzyme is resistant to the echinocandins. Using an in vitro (1,3)-β-glucan synthase activity assay, this possibility was ruled out: C. neoformans (1,3)-β-glucan synthase activity was determined to be very sensitive to the echinocandins, with an apparent caspofungin \( K_i \) of 0.17 ± 0.02 (mean ± standard deviation) \( \mu \text{M} \) and cilofungin \( K_i \) of 22 ± 5.7 \( \mu \text{M} \) (114). Consistent with these results, caspofungin has been shown to reduce both β-(1,3) and β-(1,6) glucan linkages in the C. neoformans cell wall (65). Other possible explanations for the resistance to echinocandins include rapid efflux from the cell, for example, by multidrug transporters, or the echinocandins could be degraded, either extracellularly or intracellularly.

A role for calcineurin in mediating echinocandin resistance is another attractive possibility given that calcineurin regulates the expression of (1,3)-β-glucan synthase in S. cerevisiae. Consistent with this thinking, the calcineurin inhibitor FK506 was found to have synergistic activity with caspofungin; the FK506 action was mediated via FKBP12-dependent inhibition of calcineurin (48). These results suggest that, as with S. cerevisiae, calcineurin may play a role in (1,3)-β-glucan synthase function and further suggest possibilities for synergistic drug combinations. Notably, caspofungin was also shown to have synergistic activities with amphotericin B and fluconazole against C. neoformans in vitro (68).

The possible connections between cellular stress responses and resistance to cell wall biosynthesis inhibitors extend beyond calcineurin to the cell wall integrity MAPK Mpk1. C. neoformans Mpk1 was found to be activated by phosphorylation in response to perturbations of cell wall biosynthesis by caspofungin, as well as by the chitin synthase inhibitor nikkomycin Z (92). Furthermore, mutants lacking Mpk1 demonstrated enhanced susceptibility to caspofungin and nikkomycin Z. Inhibition of calcineurin function by FK506 was also found to activate Mpk1 and result in the induction of FKS1. In the absence of calcineurin, Mpk1 partially protected cells from caspofungin-induced cell wall stress, suggesting that calcineurin and Mpk1 play complementary roles in regulating cell integrity in C. neoformans (92). The results of these studies suggest that pharmacological inhibition of the cell integrity pathway could synergistically enhance the activity of antifungals that target the cell wall.

An antifungal that is interesting in the context of stress responses and signaling molecules in C. neoformans is fludioxonil, which seems to target signal transduction. Fludioxonil is used as an agricultural fungicide to manage plant pathogenic fungi such as Botrytis cinerea, but its effects on fungal signaling have also been explored in C. neoformans (8, 89). Three signaling cascades have been found to influence the sensitivity of C. neoformans to fludioxonil, including those regulated by calcineurin, Mpk1, and Hog1 (89). The Hog1 MAPK pathway was found to promote sensitivity to fludioxonil: fludioxonil exposure activated the Hog1 osmosensing pathway in a drug-sensitive wild-type strain, and hog1Δ conferred resistance. In contrast, the Mpk1 MAPK pathway was found to mediate resistance to fludioxonil: mpk1Δ strains were hypersensitive to fludioxonil. Calcineurin also contributes to fludioxonil resistance given that calcineurin mutants were hypersensitive to fludioxonil and that the calcineurin inhibitor FK506 had synergistic activity with fludioxonil (89). C. neoformans uses a two-component system with multiple sensor kinases, including Tco1 and Tco2, that play overlapping and distinct roles in fludioxonil sensitivity through the Hog1 MAPK signaling cascade (8).

CONCLUSION

Antifungal resistance in the three major human pathogenic invasive fungal infections is becoming an increasing problem. Unfortunately, the variability in clinical findings mirrors the heterogeneity seen in molecular analysis of possible genetic mutation etiologies. Triazole resistance may become more common in the future with increasing use of antifungal prophylaxis and empirical therapy. A common theme among the fungal pathogens is that signaling molecules have a key role in mediating cellular stress responses. Harnessing these stress responses through pharmacological inhibition of signaling
pathways may provide the foundation for new combination therapies to enhance the efficacy of our limited armamentarium of clinically useful antifungal drugs or impede the evolution of antifungal resistance.

There appear to be striking similarities in antifungal resistance mechanisms among these three serious human pathogens that have diverged over millions of years of evolution. While antifungals act on each fungal organism in a slightly different manner, the fundamental nature of resistance, through alteration of antifungal target enzymes, drug efflux, or cellular stress responses, remains strikingly conserved. These broader questions surrounding the underlying molecular mechanisms of antifungal resistance, in the carefully studied context of clinical treatment failure, will allow the development of novel fungus-specific targets for improved clinical efficacy.

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