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Molecular Evolution of Antifungal Drug Resistance

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Abstract

The fungal pathogens *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* have transitioned from a rare curiosity to a leading cause of human mortality. The management of infections caused by these organisms is intimately dependent on the efficacy of antifungal agents; however, fungi that are resistant to these treatments are regularly isolated in the clinic, impeding our ability to control infections. Given the significant impact fungal pathogens have on human health, it is imperative to understand the molecular mechanisms that govern antifungal drug resistance. This review describes our current knowledge of the mechanisms by which antifungal drug resistance evolves in experimental populations and clinical settings. We explore current antifungal treatment options and discuss promising strategies to impede the evolution of drug resistance. By tackling antifungal drug resistance as an evolutionary problem, there is potential to improve the utility of current treatments and accelerate the development of novel therapeutic strategies.

Contents

INTRODUCTION

The serendipitous discovery of the antibacterial agent penicillin in 1929 (8) and the development of the first antifungal agents nystatin and amphotericin B in the 1950s (87) enabled the effective control of infectious disease for the first time in history. These discoveries propelled the golden era of antibiotics, which yielded a plethora of chemical matter with efficacy against diverse microorganisms. The ability to treat infections caused by pathogenic microbes was revolutionary to medical practice, with an increasing threat of infection arising from the more frequent use of invasive surgeries, chemotherapy for the treatment of cancer, and organ transplantation. However, in the past 80 years, pathogenic strains of bacteria and fungi that are resistant to most, or all, available antimicrobials are routinely isolated, eroding our capacity to effectively control infections and creating an urgent need for novel treatments for infectious disease (13, 47, 138). This need is particularly acute for antifungal therapeutics as there are currently only three classes of drugs approved for the treatment of systemic fungal infections (99). This paucity of antimicrobials demands a concerted effort toward the discovery of novel therapeutics for infectious disease.

With the revolutions in modern medicine that accompanied the golden age of antibiotic discovery, including advances in cancer chemotherapy and transplantation surgery, coupled with the HIV epidemic, the past several decades have witnessed a surge in immunocompromised individuals. Consequently, invasive fungal infections have transitioned from a rare curiosity to a major cause of human morbidity and mortality in both developed and developing countries. Fungi are now the causative agents of billions of infections worldwide, resulting in approximately 1.5 million deaths per year, numbers akin to prominent bacterial or protozoan pathogens such as those causing tuberculosis or malaria (14, 15). Species of *Aspergillus*, *Candida*, and *Cryptococcus* are the predominant causative agents of fungal infections in humans, accounting for over 90% of mycotic

deaths (14, 15). *Candida albicans* is a natural member of the mucosal microbiota, but in the last several decades it has also served as the leading causal agent of life-threatening invasive infections in North America and Europe, with mortality rates approaching 40% despite treatment (94). There has also been a stark rise in infections caused by *Candida* species that are intrinsically resistant to azole antifungals, including *Candida glabrata*, *Candida krusei*, and *Candida auris* (18, 25, 94), prompting the Centers for Disease Control and Prevention to classify azole-resistant *Candida* species as a serious threat to human health (22). Cryptococcosis caused by *Cryptococcus neoformans* and *Cryptococcus gattii* infects over 1 million individuals annually, with mortality rates as high as 70% in developing countries (14). Moreover, these species are recalcitrant to treatment with the newest antifungal class, the echinocandins, limiting therapeutic options. Finally, *Aspergillus fumigatus* is a significant cause of infection for individuals undergoing solid organ transplantation and those suffering from neutropenia. More than 200,000 cases of invasive aspergillosis are reported each year, with mortality rates of 100% if left untreated (14). Each of these chief opportunistic invaders has become a dominant threat to human health, and the emergence of drug-resistant strains has been reported in all species.

Given the implications of fungal drug resistance for human health, it is of central importance to understand the molecular mechanisms that govern antifungal drug resistance in order to thwart resistance. This review explores the mechanisms by which antifungal drug resistance evolves on timescales that are observed in experimental populations, laboratory settings, or human hosts undergoing treatment. We also highlight the current treatment options that are employed to treat fungal infections and discuss promising strategies to impede the evolution of drug resistance.

Resistance: the ability of a pathogen to thrive and reproduce in the presence of an antimicrobial; often attributed to genetic mutations

Ergosterol: the main sterol in the fungal cell membrane responsible for structural and regulatory membrane features, such as fluidity and permeability

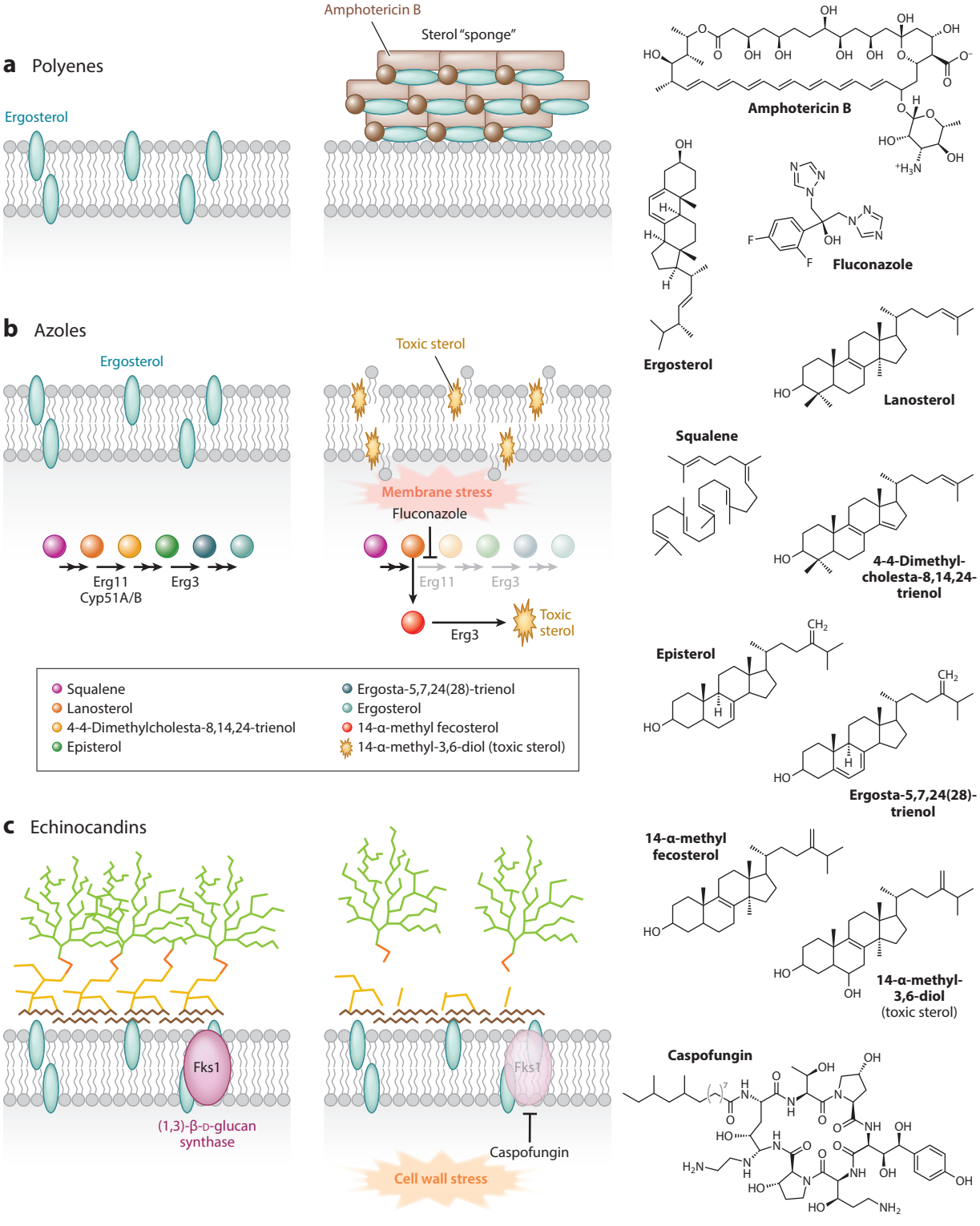
AGENTS OF SELECTION

Polyenes

Since their discovery in the 1950s, the polyenes have been employed as major fungicidal agents against several species of *Aspergillus*, *Candida*, and *Cryptococcus* (108). The polyenes are amphipathic, natural products. For decades it was thought these molecules elicited their toxic effects by intercalating into ergosterol-containing membranes to form membrane-spanning channels that caused leakage of cellular components and, ultimately, cell death (87). However, recent detailed structural and biophysical studies highlighted that polyenes bind and extract the major fungal membrane sterol ergosterol from cell membranes (**Figure 1*a***), preventing ergosterol from serving its many essential cellular functions (2). Given that polyenes directly target this central molecular node in yeast cellular physiology, clinically relevant resistance to the polyenes is extremely rare (128). Despite their potent killing activity, the use of polyenes in the clinic is limited by severe host toxicity due to the close structural relationship between ergosterol and the mammalian membrane sterol cholesterol (48, 108). Nephrotoxicity is a common side effect of polyene treatment, which lipid-based drug delivery systems have helped to partially mitigate (48, 53). Currently, amphotericin B is used to treat severe systemic fungal infections where alternative treatments are unavailable or unsuccessful (39, 88, 129). For example, amphotericin B is used in combination with the antimetabolite pyrimidine flucytosine, which inhibits DNA and RNA synthesis, as the primary treatment for cryptococcal meningitis (39). The development of alternative amphotericin B preparations and polyene derivatives with improved therapeutic indices is ongoing (53, 99).

Azoles

For over two decades, the azoles have been the most widely deployed class of antifungal drugs, due to their broad spectrum of activity, favorable safety profile, and bioavailability (99, 101). The


a Polyenes
Ergosterol
Amphotericin B
Sterol "sponge"
Amphotericin B
b Azoles
Ergosterol
Erg11
Cyp51A/B
Erg3
Toxic sterol
Membrane stress
Fluconazole
Erg11
Erg3
Erg3
Toxic sterol
Fluconazole
Ergosterol
Lanosterol
Squalene
4-4-Dimethyl-cholesta-8,14,24-trienol
Episterol
Ergosta-5,7,24(28)-trienol
Squalene
Lanosterol
4-4-Dimethylcholesta-8,14,24-trienol
Episterol
Ergosta-5,7,24(28)-trienol
Ergosterol
14-α-methyl fecosterol
14-α-methyl-3,6-diol (toxic sterol)
c Echinocandins
Fks1
(1,3)-β-D-glucan synthase
Fks1
Caspofungin
Cell wall stress
14-α-methyl fecosterol
14-α-methyl-3,6-diol
(toxic sterol)
Caspofungin

azoles block ergosterol biosynthesis through inhibition of the lanosterol 14-α-demethylase enzyme encoded by *ERG11* in *Candida albicans* and *Cryptococcus neoformans* and *cyp51A* and *cyp51B* in *A. fumigatus* (108) (**Figure 1*b***). This results in the depletion of ergosterol and the buildup of a toxic membrane sterol produced by Erg3, which exerts a membrane stress when incorporated into the fungal membrane and ultimately inhibits proliferation (108) (**Figure 1*b***). The clinically approved azoles include fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole and have mainly fungistatic activity against yeasts, such as *Candida* and *Cryptococcus* species (79, 99, 101). As an exception, voriconazole displays fungicidal activity against *A. fumigatus* and outperforms amphotericin B as a primary therapy for invasive aspergillosis (55, 78). However, the typically fungistatic nature of the azoles that allows for the survival of fungal populations coupled with the extensive use of azoles as prophylactics (88, 90) has resulted in widespread resistance to azoles (22, 99).

Echinocandins

The newest class of antifungal to reach the clinic is the echinocandins, which includes the clinically approved drugs micafungin, caspofungin, and anidulafungin (92). The echinocandins are large, semisynthetic lipopeptide molecules that noncompetitively inhibit the (1,3)-β-D-glucan synthase enzyme encoded by *FKS1* (108) (**Figure 1*c***). Targeting Fks1 disrupts the synthesis of the major cell wall biopolymer (1,3)-β-D-glucan, resulting in loss of cell wall integrity and imparting a severe cell wall stress on the fungus (92). The echinocandins are fungicidal against most *Candida* species and display either cidal or static activity against *A. fumigatus*. However, the activity spectrum of these molecules is limited, with no clinical efficacy against *Cryptococcus* species (40). Due to their impressive safety profile and potent killing activity, the echinocandins have emerged as the first-line therapy for the treatment of systemic candidiasis (88). All echinocandins to date suffer from low oral bioavailability, and thus the development of echinocandin analogs and novel glucan-targeting molecules with improved oral absorption is currently under way (99). Although the overall prevalence of echinocandin resistance remains low, their increased use for prophylaxis and prolonged treatment regimens has resulted in an increased incidence of clinically relevant resistance (92). Echinocandin-resistant *C. glabrata* infections have risen drastically and now pose a significant concern given that many isolates display cross-resistance to the azoles (93).

DIVERSITY IN DRUG RESISTANCE

The evolution of drug resistance is an ancient and ubiquitous phenomenon, as microorganisms adapt to outcompete and survive in their natural environments (37, 137). Variation in drug resistance can be observed at multiple levels. At the species level, fungi can differ in their

Figure 1

Mechanisms of action of clinically relevant antifungal drugs. (*a*) Polyenes such as amphotericin B exist primarily in the form of large, extramembranous aggregates that extract ergosterol from lipid bilayers. The chemical structures of amphotericin B and ergosterol are shown on the right. (*b*) The azoles function by targeting the ergosterol biosynthetic enzyme lanosterol demethylase, encoded by *ERG11* (*Candida albicans* and *Cryptococcus neoformans*) or *cyp51A* and *cyp51B* (*Aspergillus fumigatus*), causing a block in the production of ergosterol and the accumulation of a toxic sterol, 14-α-methyl-3,6-diol, produced by Erg3. This toxic sterol exerts severe membrane stress on the cell. The chemical structures of relevant sterol intermediates are shown on the right. Double arrows represent multiple chemical reactions for the synthesis of ergosterol. (*c*) Fungal cell walls are composed of (1,3)-β-D-glucans (*yellow chains*) covalently linked to (1,6)-β-D-glucans (*orange chains*) as well as chitin (*brown*), mannans (*green*), and cell wall proteins. The echinocandins act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase, encoded by *FKS1* (*C. albicans*, *C. neoformans*, and *A. fumigatus*), and thereby cause a loss of cell wall integrity and severe cell wall stress. The chemical structure of caspofungin is shown on the right. Figure adapted, with permission, from Reference 29.

inherent ability to proliferate during the stress induced by drug exposure independently of the acquisition of specific adaptive mutations, which is often referred to as tolerance (34, 99, 108). At the population level, fungi can acquire specific mutations that reduce the inhibitory effects of a drug, which is referred to as resistance. The frequency with which resistance is acquired varies dramatically by class of antifungal. For example, polyene resistance is extremely rare due to the fitness consequences associated with resistance development, including hypersensitivity to oxidative stress, febrile temperatures, and neutrophil killing (128). In contrast, resistance to the azoles is far more prevalent due to their fungistatic nature that results in strong selective pressures exerted on surviving populations (1, 99). For the echinocandins, a specific type of tolerance referred to as the paradoxical effect is frequently observed, whereby growth of the fungus is restored at drug concentrations substantially higher than the reported minimum inhibitory concentration (120, 134). The mechanism that enables this growth appears to be due in part to the transcriptional upregulation of chitin synthases in *A. fumigatus* and *C. albicans* upon echinocandin exposure (120), which allows for the acquisition of mutations that confer bona fide resistance. Despite the varied rates at which resistance to the different antifungal classes emerges, resistance to all antifungals has been documented in both the laboratory and clinic (108).

Tolerance: the inherent ability of an organism to proliferate in the presence of an antimicrobial independently of specific adaptive mutations

Fitness: the extent to which an individual contributes genes to future generations

Paradoxical effect: the ability of a fungal strain to grow at drug concentrations above the minimum inhibitory concentration; also known as trailing growth

Stress response: various mechanisms that enable microorganisms to survive adverse and fluctuating conditions in their immediate surroundings

Variation in response to antifungals exists between both distantly related and closely related species, as well as between strains within a species. For example, although fluconazole is one of the most frequently deployed antifungals for treating *Candida* and *Cryptococcus* infections, *A. fumigatus* is intrinsically resistant due to specific residues in the drug target Cyp51A, necessitating the use of voriconazole as a therapeutic option (69, 99). Perhaps most alarming is that species of *Cryptococcus*, which are responsible for greater than 620,000 deaths per year, are resistant to the echinocandins, limiting treatment options to drugs that target ergosterol or its biosynthesis (14, 40). It remains elusive how *Cryptococcus neoformans* can tolerate echinocandin concentrations that are typically inhibitory, given that the target (1,3)-β-D-glucan synthase is essential and inhibited by the echinocandins in vitro (72). Variation in susceptibility to antifungals also occurs between closely related fungal species. Up to 20% of *Candida glabrata* strains are intrinsically resistant to azoles and even susceptible strains rapidly acquire resistance, prompting clinicians to recommend echinocandin drugs as a first-line therapy to treat a range of candidiasis (88). Other *Candida* species such as *C. lusitaniae* and *C. guillermondii* are intrinsically resistant to amphotericin B (95), and *C. parapsilosis* and *C. glabrata* show greater intrinsic resistance to the echinocandins (5). Most alarming are the recent global outbreaks of *C. auris*, which displays elevated resistance to all antifungal drug classes, leaving no effective therapeutic options (18, 25). Variations in susceptibility profiles are also observed for *Aspergillus* species in response to the polyenes. Overall, *A. flavus* and *A. terreus* are capable of tolerating higher concentrations of amphotericin B compared to other *Aspergillus* species (4), due to the differences in the management of oxidative damage (11).

Variations in resistance phenotypes are also observed within a population of cells of a single fungal species or strain, with such variation often being transient. For example, surface-associated microbial communities, or biofilms, represent a clinically important example of heterogeneity in drug resistance (49). Relative to their planktonic counterparts, biofilms exhibit remarkable alterations in the cellular physiology of a community that are associated with extreme resistance to antifungal therapy. The glucan matrix that surrounds the microbial population contributes to the elevated drug resistance phenotype by acting as a physical barrier between drug and microbe, but other factors such as alterations in efflux pump expression, changes in cell membrane and wall composition, and global changes in cellular stress response profiles collectively confer the elevated drug resistance phenotype (77, 122). Biofilms also offer a refuge from antifungal drugs and the host immune response, which facilitates the development of persister cells able to tolerate high concentrations of drug (63). Persisters are a phenotypic variant that arises in the biofilm

community and are thought to be a major factor affecting the recalcitrance of fungal biofilm infections to treatment (63).

The phenomenon of heteroresistance is another fascinating example of variation in drug susceptibility within a population. In *Cryptococcus neoformans*, *Cryptococcus gattii*, and *Candida albicans*, single cells are capable of giving rise to progeny with heterogeneous resistance phenotypes, with a small but consistent subset of progeny being highly resistant to the azoles (17, 115, 125). This phenomenon allows populations to adapt to increasing concentrations of azoles in a stepwise manner, with the original susceptibility being restored after passage in the absence of drugs (115). The molecular mechanism governing this response in *C. neoformans* involves the acquisition of a disomy on chromosome 1, which harbors the genes for the azole target *ERG11* and the efflux transporter *AFR1* (117). Such phenomena have been observed in both laboratory and clinical settings (115, 116), representing an intrinsic adaptive mechanism for survival during azole stress.

Virulence: the pathogenicity of an organism or its ability to cause disease

ATP-binding cassette (ABC) transporters: a protein family that uses energy provided by the hydrolysis of ATP to transport substrates across membranes

ADAPTIVE MECHANISMS

The canonical mechanisms by which antimicrobial resistance evolves are drug target alteration or overexpression, reduction of intracellular accumulation of the drug, or activation of cellular stress response pathways. These mechanisms do not occur in isolation, but instead often collectively contribute to drug resistance of clinical isolates.

Drug Target Alteration and Overexpression

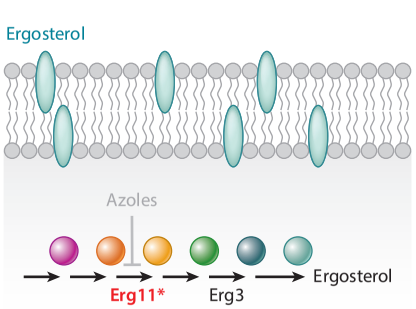
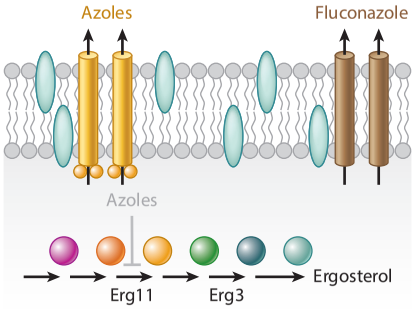
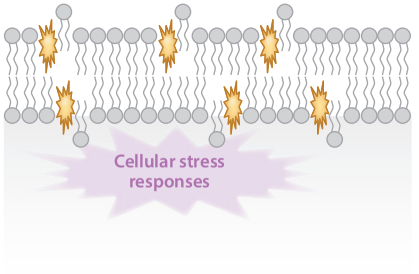
One of the most common mechanisms by which microorganisms evolve resistance to antimicrobials is through mutations in the drug target gene that reduce drug binding and efficacy (**Table 1**). In *C. albicans*, this is a frequent mechanism by which resistance to the azoles evolves. Numerous mutations in the azole target gene *ERG11* have been identified in clinical isolates of *C. albicans*, and these are often found in hot-spot regions adjacent to the enzyme active site (75). As *C. albicans* is a diploid organism, mutations in *ERG11* are often followed by a loss of heterozygosity, thereby conferring higher levels of azole resistance through increased dosage of the resistance allele (27). In *C. neoformans* and *A. fumigatus*, mutations in the drug target gene are also often implicated in azole resistance (41, 73, 100, 126). For the echinocandins, resistance primarily occurs through mutations in the drug target gene, often clustered in two distinct regions (89, 91) (**Table 1**). For *C. albicans*, mutations in *FKS1* are often followed by a loss of heterozygosity leaving two mutant alleles with decreased affinity for the antifungal (83).

In addition to mutations that reduce the affinity of a target for its cognate inhibitor, increased expression of a drug target can also confer resistance. For example, the *C. albicans* transcriptional activator Upc2 regulates the expression of ergosterol biosynthesis genes, including *ERG11*, in response to azole exposure, with gain-of-function mutations in *UPC2* leading to constitutive overexpression of *ERG11* in azole-resistant isolates (43, 59, 80). In *C. neoformans*, expression of *ERG11* is governed by a distinct sterol regulatory element-binding protein (SREBP)-like transcription factor, Sre1, which regulates *ERG11* expression in response to azoles. Sre1 pathway mutants also have important roles in virulence (9, 23). In *A. fumigatus*, a similar SREBP homolog, SrbA, is required for sterol regulation in response to antifungal drugs and has been implicated in virulence in animal models (135).

Reducing Intracellular Drug Accumulation

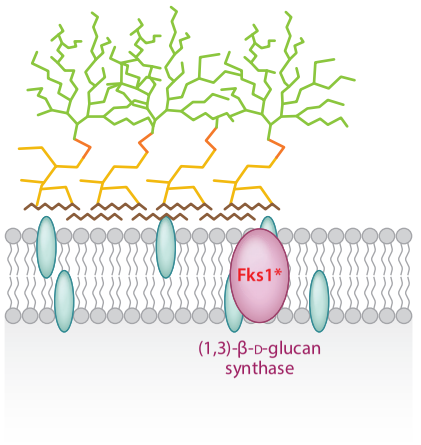
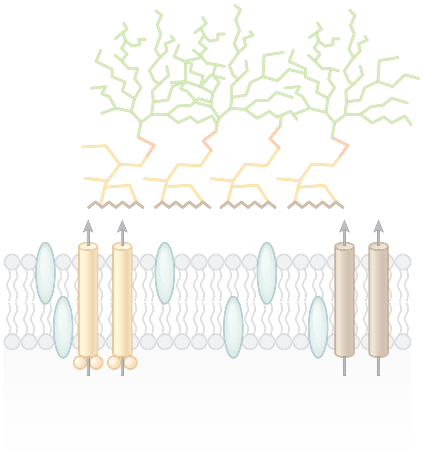
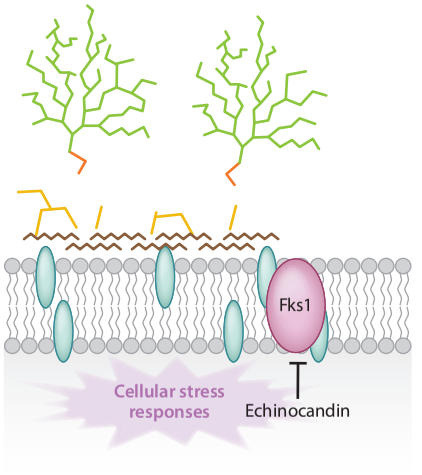
Another way in which microorganisms evolve resistance to toxic compounds is by reducing accumulated intracellular drug by activation of efflux pumps (**Table 1**). ATP-binding cassette (ABC)

Table 1 Adaptive mechanisms of drug resistance[a]

Target alteration or overexpression	Pump overexpression	Cellular stress responses
Azoles[b]		
		
Candida albicans		
Mutations in *ERG11* and *UPC2* Loss of heterozygosity Isochromosome i(5L)	Overexpression of Cdr1, Cdr2, and Mdr1 Mutations in *TAC1* and *MRR1* Loss of heterozygosity Isochromosome i(5L)	*erg3* mutations confer stress response–dependent resistance Mediators of stress response pathways include Hsp90, Sgt1, calcineurin, KDACs, PKC, and TOR
Cryptococcus neoformans		
Mutations in *ERG11* Overexpression of Erg11 due to chromosome duplication	Overexpression of Arf1 due to chromosome duplication	Mediators of stress response pathways include ATPases and the oxygen-sensing pathways
Aspergillus fumigatus		
Mutations in *cyp51A* Overexpression of *cyp51A*	Overexpression of AtfR, Mdr3, and Mdr4	

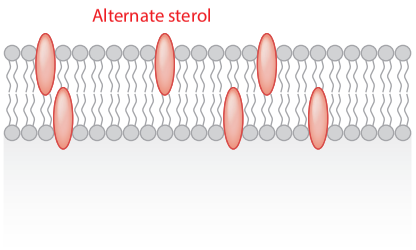
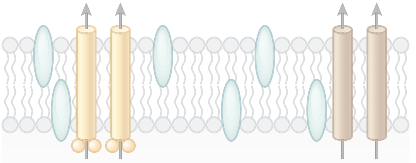
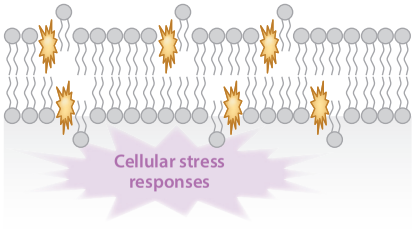
(*Continued*)

Table 1 (*Continued*)

Target alteration or overexpression	Pump overexpression	Cellular stress responses
Echinocandins[c]		
		
Candida albicans		
Mutations in *FKS1*		Mediators of stress response pathways include Hsp90, calcineurin, KDACs, PKC, and HOG
Aspergillus fumigatus		
Mutations in *FKS1* increase echinocandin resistance in vitro; not a mechanism commonly found in the clinic		Mediators of stress response pathways include Hsp90, calcineurin, Ras, and the unfolded protein response

(*Continued*)

Table 1 *(Continued)*

Target alteration or overexpression	Pump overexpression	Cellular stress responses
Polyenes[d]		
		
Candida albicans		
Loss-of-function mutations in ergosterol biosynthesis genes confer resistance		Mediators of stress response pathways include Hsp90

[a]Dimmed images indicate those mechanisms that do not play a key role in resistance to the specific drug class. Asterisks (*) indicate a mutation or altered protein that is blocking drug binding.
[b]Resistance to azoles can arise through multiple mechanisms in pathogenic fungi, such as through the mutation or overexpression of the azole target Erg11 (*Candida albicans* and *Cryptococcus neoformans*) or *cyp51A* (*Aspergillus fumigatus*), from the upregulation of two classes of efflux pumps that remove the drug from the cell, or through cellular alterations that mitigate drug toxicity or enable responses to drug-induced stress. Mutations in *FKS1*, which encodes the (1,3)-β-D-glucan synthase catalytic subunit, are the most prevalent mechanism of echinocandin resistance. Resistance phenotypes are modulated by cellular stress response pathways.
[c]Resistance to echinocandins primarily evolves through mutation in the drug target Fks1 and through the activation of mediators of cellular stress response pathways.
[d]Resistance to polyenes primarily involves depletion of the target ergosterol due to loss-of-function mutations in ergosterol biosynthesis genes, but stress response pathways governed by Hsp90 are also crucial for polyene resistance.
Abbreviations: HOG, high osmolarity glycerol; KDAC, lysine deacetylase; PKA, protein kinase A; PKC, protein kinase C; TOR, target of rapamycin. Adapted from Reference 29.

transporters Cdr1 and Cdr2 are clinically important efflux pumps that are regulated by the transcriptional activator Tac1 in *C. albicans* (108). Several *TAC1* gain-of-function mutations that cause a constitutive upregulation of *CDR1* and *CDR2* have been reported in azole-resistant *C. albicans* clinical isolates (28). Akin to mutations in *ERG11* and *UPC2*, loss of heterozygosity at the *TAC1* locus confers higher levels of resistance; Tac1 acts as a homodimer and two hyperactive *TAC1* alleles confer higher expression of efflux pumps (28). In *C. neoformans* and *A. fumigatus*, the ABC transporters responsible for azole efflux are Afr1 and AtrF, respectively (108). In addition to the ABC transporters, the major facilitator class efflux pump Mdr1 can transport fluconazole from *C. albicans* (131). The multidrug resistance regulator Mrr1 is the transcription factor that orchestrates *MDR1* expression, and this transcription factor appears to be coordinately upregulated with *MDR1* in drug-resistant clinical isolates (81). *A. fumigatus* also encodes four Mdr-like pumps, Mdr1–4, with roles in azole efflux (108, 124). Unlike the azoles, upregulation of efflux pumps plays a negligible role in echinocandin resistance.

As an alternative to increased efflux, organisms can reduce intracellular drug accumulation by reducing drug import. In *C. albicans* and *C. neoformans*, azoles are imported into the cell through an energy-independent facilitated diffusion mechanism (74). Recent studies in *A. fumigatus* have similarly identified a pH- and ATP-independent facilitated diffusion mechanism responsible for azole import (46). Identification of the proteins responsible for import will enable a more thorough investigation into the clinical impact of drug import on azole resistance.

Major facilitator class: a protein family that uses energy provided by the proton motive force of the membrane to transport substrates across membranes

Chaperone: a class of proteins that assist in the correct folding and maturation of other cellular proteins, collectively termed clients or substrates

Regulation of Stress Response Pathways

Microbes are equipped with complex circuitry that enables responses to diverse cellular stresses, including exposure to antifungal drugs (29, 34, 108). A key hub of cellular circuitry that enables antifungal resistance is the molecular chaperone Hsp90, an essential chaperone in all eukaryotes that regulates the stability and activation of diverse client proteins that have a profound impact on cellular signaling (123). Hsp90 promotes antifungal drug tolerance and the evolution of resistance to both azoles and echinocandins in species of *Candida* and *Aspergillus* (31, 33, 62, 64, 65, 113, 114) by stabilizing key regulators of cellular stress responses, including the protein phosphatase calcineurin and the terminal mitogen-activated protein kinase in the Pkc1 signaling cascade, Mkc1 (31, 62, 113) (**Table 1**). Inhibition of Hsp90, calcineurin, or Pkc1 signaling reduces azole and echinocandin resistance of isolates that evolved resistance in a human host (31, 36, 62, 113). In *Candida* species, polyene resistance is also contingent on robust Hsp90 function (128) (**Table 1**), highlighting the importance of this central cellular hub in mediating responses to diverse antifungal stresses.

Additional global regulators of cellular growth include the target of rapamycin (TOR) protein kinases, which control cellular responses to changes in nutrient availability (76, 102). Pharmacological inhibition of TOR function with the natural product rapamycin inhibits growth of diverse fungi, including *Cryptococcus neoformans*, *Candida albicans*, *A. fumigatus*, and *Penicillium* species (10, 35, 136), and abrogates *erg3*-mediated azole resistance in *C. albicans* (97). Recently, the natural product beauvericin, which potentiates azole activity against diverse fungal pathogens, blocks the emergence of drug resistance, and renders antifungal-resistant pathogens responsive to treatment in mammalian infection models, was shown to act in part through inhibition of TOR signaling in both *Saccharomyces cerevisiae* and *C. albicans* (111, 112). Inhibition of TOR signaling ultimately compromises Hsp90 function. Beauvericin also potentiates azole activity by inhibition of efflux pumps (111, 112). Thus, cellular regulators governing stress response signaling provide important targets to overcome fungal drug resistance (**Table 1**).

Genomic Plasticity

Fungal species are capable of adapting to diverse environmental perturbations due in part to their remarkable genomic plasticity. The acquisition of aneuploidies facilitates the emergence of resistance to antifungal drugs due to an increased dosage of specific resistant determinants. Studies examining genome composition of azole-resistant *C. albicans* isolates derived from both laboratory and clinical settings unveiled a myriad of aneuploidies, the most common of which is a duplication of the left arm of chromosome 5 with the production of an isochromosome termed i(5L) (104–106). The formation of aneuploidies in response to fluconazole stress occurs as a consequence of abnormal cell cycle progression in which mother and daughter cells fail to separate after chromosome segregation (54). The aberrant chromosome segregation dynamics in these cell types produces progeny with double the normal number of chromosomes, leading to an increased prevalence of aneuploidy in the population (54). For those resistant isolates containing i(5L), elevated azole resistance is due to an increased dosage of both *ERG11* and *TAC1*, which are located in this region (105). The formation of i(5L) is also often coupled with a recombination event that results in a loss of heterozygosity at this locus, rendering mutations acquired at the *ERG11* or *TAC1* loci homozygous and thus further contributing to the increase in azole resistance phenotypes (27). Although certain chromosomal duplications such as i(5L) are relatively stable, studies suggest that other aneuploidies acquired in the clinic are more transient and not consistently associated with stable increases in resistance (51).

In addition to the acquisition of specific aneuploidies, changes in copy number of the entire genome may also prove beneficial for the evolution of drug resistance. Through elegantly designed experiments, polyploidy in the model yeast *S. cerevisiae* was shown to result in significantly faster adaptation in a carbon-poor environment, which was primarily driven by higher rates of beneficial mutations with large fitness effects (107). The relevance of polyploidy in the evolution of antifungal drug resistance has only begun to be explored.

ELUCIDATING MECHANISMS OF DRUG RESISTANCE

A comprehensive appreciation of mechanisms of drug resistance requires multiple approaches. One powerful approach involves the analysis of fungal isolates from an infected individual over time. This provides the most direct clinical relevance but is often constrained by small sample sizes and the inability to control population parameters such as the number of generations, effective population size, and genotype of the initial susceptible strain. This makes it challenging to identify the selective pressures that directly contribute to the evolution of resistance. For these reasons, in vivo studies of the evolution of antifungal resistance are frequently complemented by in vitro experimental evolution counterparts. Below, we focus on studies of the evolution of drug resistance in a human host followed by an elaboration on the methods and benefits of experimental evolution.

Evolution of Drug Resistance in the Human Host

Matched sets of clinical isolates from infected patients have provided a foundation for understanding how resistance mutations arise during the course of antifungal treatment (**Figure 2**). One of the best-characterized sets of fungal isolates illustrates the concept of progressive drug resistance, or resistance that increases over time as sequential isolates accumulate multiple resistance mutations (131). This set of 17 isolates of *C. albicans* was collected over two years from an HIV-infected patient who was receiving azole treatment for recurrent oropharyngeal candidiasis. The initial

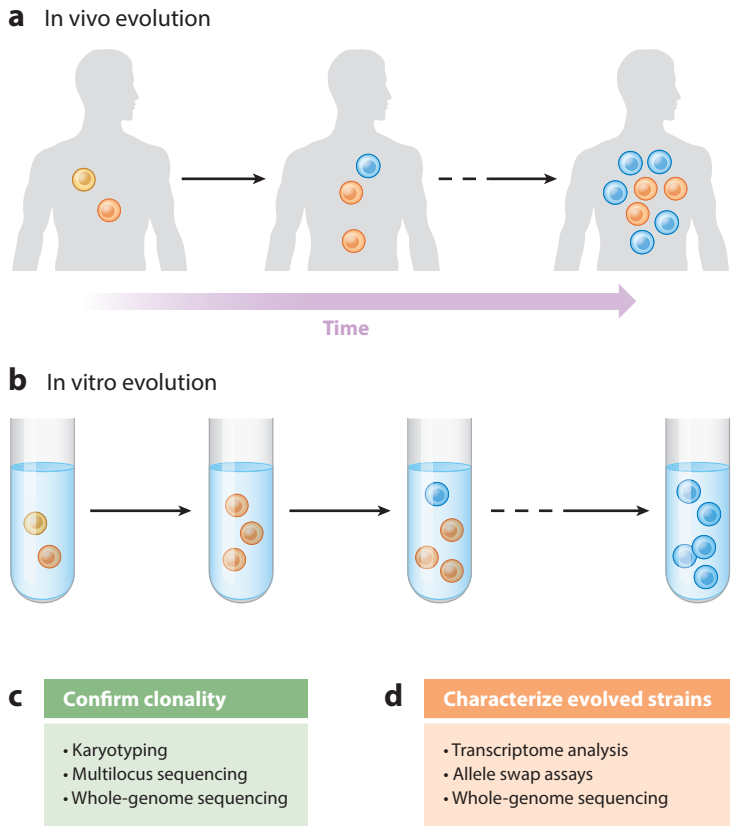


Figure 2

Elucidating mechanisms of drug resistance. (*a*) Evolution of drug resistance in the human host. The progressive accumulation of resistance mutations in clinical isolates exemplifies how resistance often develops in a stepwise fashion, with high-level resistance due to the combination of multiple mechanisms. Those strains harboring a fitness advantage (*blue*) proliferate and outcompete other strains in the human host (*yellow* and *orange*). (*b*) Experimental evolution of drug resistance. Cells are passaged by serial dilution of a stationary-phase culture into fresh medium containing a concentration of antifungal that is inhibitory but not lethal. This process is repeated until a sufficient number of generations have occurred for resistance to evolve. (*c*) Strategies employed to confirm resistant strains isolated from the clinic or experimental populations are clonal and contain the same resistance mutations. (*d*) Strategies used to characterize evolved resistance isolates and determine the molecular mechanism governing resistance.

isolate in the series was susceptible to fluconazole treatment, but by the end of the two-year period, the strain had acquired a level of resistance 200-fold higher than that of the original isolate. Mutations acquired during the sampling period were identified in genes encoding the drug target Erg11, transcriptional regulator Upc2 that causes overexpression of *ERG11*, and transcriptional regulator Tac1 that causes overexpression of *CDR1* and *CDR2*, which encode efflux transporters (59, 131–133). Notably, overexpression of the efflux pump Mdr1 was also observed in these clinical isolates (131). The progressive accumulation of resistance mutations in these isolates exemplifies how resistance often develops in a stepwise fashion, with high-level resistance being attained due to the combination of multiple mechanisms.

Experimental Evolution of Drug Resistance

A powerful approach gaining prominence in the study of antifungal resistance is experimental evolution, which involves the propagation of populations under laboratory conditions such that new traits can evolve and be monitored in real time (**Figure 2**). Typically, cells are passaged by serial dilution of a stationary-phase culture into fresh medium containing a concentration of antifungal that is inhibitory but not lethal. It is crucial that the population size at each transfer is large enough that beneficial mutations that arise are not lost to random drift (45). This process is repeated until a sufficient number of generations has occurred for resistance to evolve. Experimental evolution studies have also been performed with *C. albicans* in a mouse model host (50). Passage in a mouse led to different fungal population dynamics compared to that observed with in vitro evolution, including slower growth rates as well as increased genotypic and phenotypic variation in the mouse (50).

In vitro evolution experiments can provide a simple model for how drug resistance emerges in the host. The capacity for direct control over experimental parameters affords greater power for identifying factors that give rise to drug resistance. Furthermore, experiments are easily replicated, so the sample size can be much greater than what is achieved with clinical isolates from patients. With recent advances in sequencing technology, experimental evolution can be coupled with genome sequencing, providing a global view of adaptive mutations (56, 58). In addition, RNA-sequencing technology can enable the identification of genes that are specifically overexpressed in drug-resistant isolates, thereby illuminating mechanisms of resistance. The primary limitation of in vitro evolution experiments is that laboratory conditions will never be able to recapitulate the complexity of growth environments experienced in a human host. In the human body, organisms experience diverse challenges, including variation in microenvironments, nutrient limitation, spatial structure, and competition with other microorganisms. Despite these limitations, studies with *C. albicans* and *A. fumigatus* have highlighted that drug-resistant lineages evolved in vitro often acquire mechanisms of resistance that are also observed in clinical isolates (32, 38, 71). Aneuploidies observed in clinical isolates are also frequently identified in lineages that were evolved in vitro, with antifungal resistance attributable to increased gene dosage of resistance determinants (106). Thus, experimental evolution provides a powerful approach to elucidate the mechanisms by which drug resistance arises.

Fitness Effects of Resistance Mutations

An appreciation of the fitness consequences of resistance mutations is crucial to predicting the emergence and spread of resistance. There is precedent for resistance mutations being associated with a fitness cost, leading to a competitive disadvantage in the absence of drug. This can be due to the pleiotropic effects of resistance mutations, as would be expected with transcriptional regulators that control the expression of azole resistance determinants such as *UPC2*, *TAC1*, and *MRR1*, as well as many additional genes in the genome (108). Recent analysis of the series of 17 clinical isolates obtained from an HIV-infected individual (131) revealed that the later clinical isolates that had acquired mutations in *UPC2*, *ERG11*, and *TAC1* maintained azole resistance despite inhibition of Hsp90 (31, 58) but had fitness defects in the absence of drug or in the presence of alternate stressors (58). Notably, fitness defects can be ameliorated by the accumulation of compensatory mutations (58). Compensatory evolution is well established in bacteria (3, 103) but less well explored in fungi (114). An appreciation of the spectrum of compensatory mutations that

mitigate the cost of resistance may provide a foundation from which to predict and prevent the spread of drug resistance.

THWARTING THE EVOLUTION OF DRUG RESISTANCE

Combination Therapy

The scarcity of novel antifungal drugs in development coupled with the emergence of resistance to all clinically employed antifungals necessitates the identification of strategies to enhance the efficacy of antifungals and block the emergence of drug resistance. Combination therapy is a promising approach to prolong the life span of existing antifungals. Drug combinations have the potential to impede the evolution of drug resistance through several mechanisms. Combining two agents can produce an increased killing effect, reducing the pathogen population size and thus the probability of acquiring resistance mutations (1, 57, 119). Additionally, where each antifungal agent has a distinct mechanism of action, the likelihood of evolving resistance to each agent individually is less than the probability of evolving resistance to either agent alone (1, 57, 119). The enhanced inhibitory effects of combinations also allow for lower individual drug dosage and reduced treatment length, minimizing host toxicity (1, 57, 119). Interestingly, specific drug combinations also have the potential to reverse drug resistance through a process called selection inversion (7), which was recently demonstrated using tetracycline-resistant *Escherichia coli* (121). Currently, combination therapies are the standard of care for the treatment of complex infectious diseases such as malaria and AIDS/HIV; however, drug combinations remain relatively underexplored as antifungal therapies.

The selection of drugs for combination therapy can be accomplished through several strategies, based either on prior knowledge of resistance mechanisms or on systematic screening for effective combinations. As highlighted above, fungal stress response pathways are required for survival in response to cellular stress imposed by antifungal drugs, and thus targeting these pathways provides a powerful approach to improve drug efficacy (30, 127). A variety of stress response inhibitors show therapeutic benefits in combination with current antifungals, including inhibitors of Hsp90 (31, 33), calcineurin (36), protein kinase C (62), and TOR signaling (111, 112). Directly targeting established resistance determinants, as is employed with the combination of β-lactam antibiotics and β-lactamase inhibitors to treat bacterial infections (42, 60), is another example of the intelligent selection of drug combinations. Recently, the small molecule iKIX1 was found to abrogate azole resistance in *Candida glabrata* in vivo by disrupting the interaction between the mediator complex and the Pdr5 efflux pump transcriptional activator Pdr1, whose constitutive activation confers azole resistance (84). Recent studies also suggested that targeting the formation of aneuploidies associated with drug resistance in *Candida albicans* may provide a viable therapeutic strategy. By designing an evolutionary trap that targets both genotypic diversity and fitness, azole resistance was regressed in a strain of *C. albicans* harboring the i(5L) aneuploidy (24). This highlights the potential of targeting drug resistance regulators in combination with antifungals to treat systemic fungal disease.

A complementary method for identifying combination therapies is through large-scale systematic screens of compound libraries to identify molecules that potentiate the activity of current antifungals. In recent years, the use of large compound collections for chemical biology screens has identified inhibitory drug combinations against *S. cerevisiae*, *C. albicans*, and *Cryptococcus neoformans* (16, 96, 98, 118). In one such example, researchers screened a panel of approximately 3,600 bioactives for synergy with six antifungals against diverse fungal species, allowing for the identification of approximately 1,550 combinations that abrogate fungal growth in at least one condition (98).

Another screen of 1,280 active compounds identified potent synergy between echinocandins and the broad-spectrum chelator diethylenetriaminepentaacetic acid against echinocandin-resistant *C. albicans* both in vitro and in a mouse model of candidiasis (96). These studies highlight the utility of high-throughput screening to identify efficacious combinations to treat systemic fungal infections.

Targeting Virulence

A complementary approach to identify new therapeutic strategies to combat fungal infections is to target proteins that are required for pathogen growth in the host or for virulence. Although the prospective utility of targeting nonessential genes has only recently been appreciated, potential benefits include dramatically expanding the number of cellular targets that could be exploited by novel chemical scaffolds, minimizing negative impacts on the host mycobiome, and reducing selection pressure for the evolution of drug resistance (26, 52). In fungi, key virulence targets have been identified that may provide promising therapeutic targets. For example, the pathogenic prowess of *C. albicans* is dependent on its ability to transition between yeast and filamentous morphologies (61, 85). Most mutants that grow exclusively as yeast are avirulent in mouse models of systemic candidiasis (68, 70), as are mutants that grow exclusively as filaments (6, 12, 66, 82). Proof-of-principle studies have highlighted the feasibility of targeting *C. albicans* morphogenetic regulators. For example, beyond its impact on fungal drug resistance, Hsp90 is a key regulator of morphogenesis, and depletion of *C. albicans* Hsp90 attenuates virulence in a murine model of systemic infection (109, 127). In *C. neoformans*, key virulence factors include the production of a polysaccharide capsule and the deposition of laccase-synthesized melanin in the cell wall. Targeting the *C. neoformans* capsule may be feasible through the use of polysaccharide conjugate vaccines, which elicit strong antibody responses to permit the isolation of several anticapsule antibodies from mice (20, 21, 110), and some have shown promise in phase I clinical trials (67). In regard to targeting melanin, the antipsychotic drug trifluoperazine affects growth only of melanized cells in vitro (130) and reduces mortality of mice when administered daily postinfection (44). In addition, the herbicide glyphosate inhibits melanin production and prolongs mouse survival upon infection with *C. neoformans* (86). To date, most fungal virulence factors have been defined through small-scale studies; however, with the continued development of functional genomic resources, large-scale studies will enable the systematic discovery of virulence genes and thereby expand the target space for antifungal drug development.

OUTLOOK AND FUTURE PERSPECTIVES

The evolution of drug resistance in fungal pathogens remains a critical concern. That resistance has evolved to a diverse repertoire of antifungals provides a poignant illustration of the basic principle that resistance will ultimately evolve to any new antifungal or combination of antifungals used for treatment. The infamous line proclaimed by the Red Queen in *Through the Looking Glass*, "Now, here, you see, it takes all the running you can do, to keep in the same place!" (19), describes the challenge inherent in tackling antimicrobial resistance. Nonetheless, promising new screening platforms, an expansion of functional genomic resources in fungal pathogens, improved technologies for natural product drug discovery, and the exploration of combinations to treat fungal infections can all be leveraged to face this challenge. Approaching antifungal drug resistance as an evolutionary problem is crucial to combating resistance and accelerating the development of much-needed novel therapeutic strategies.

SUMMARY POINTS

1. There are a limited number of antifungal drugs for the treatment of invasive fungal infections that impact millions of individuals annually.
2. Resistance to all available antifungal drug classes, either intrinsic or acquired, has been documented in both laboratory and clinical settings.
3. Drug target alterations are a prevalent cause of resistance to the three most widely deployed classes of antifungal drugs in the leading fungal pathogens of humans.
4. Overexpression of multidrug transporters is a common mechanism of resistance to the azoles and often achieved through gain-of-function mutations in transcription factors controlling their expression and/or the acquisition of aneuploidies.
5. Cellular stress responses governed by Hsp90, calcineurin, protein kinase C, and TOR protein kinase modulate critical signaling pathways in response to drug-induced stress.
6. In vivo studies of clinical isolates monitoring the evolution of antifungal resistance complemented by in vitro experimental evolution counterparts offer powerful insights into the mechanisms that govern antifungal resistance.
7. Combination therapy can increase the effectiveness of current antifungals and minimize the emergence of antimicrobial resistance.
8. A thorough understanding of the mechanisms by which antifungal resistance is acquired is critical for developing novel and effective therapeutic strategies.

DISCLOSURE STATEMENT

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