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Antifungal drug resistance: evolution, mechanisms and impact

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Microorganisms have a remarkable capacity to evolve resistance to antimicrobial agents, threatening the efficacy of the limited arsenal of antimicrobials and becoming a dire public health crisis. This is of particular concern for fungal pathogens, which cause devastating invasive infections with treatment options limited to only three major classes of antifungal drugs. The paucity of antifungals with clinical utility is in part due to close evolutionary relationships between these eukaryotic pathogens and their human hosts, which limits the unique targets to be exploited therapeutically. This review highlights the mechanisms by which fungal pathogens of humans evolve resistance to antifungal drugs, which provide crucial insights to enable development of novel therapeutic strategies to thwart drug resistance and combat fungal infectious disease.

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Introduction

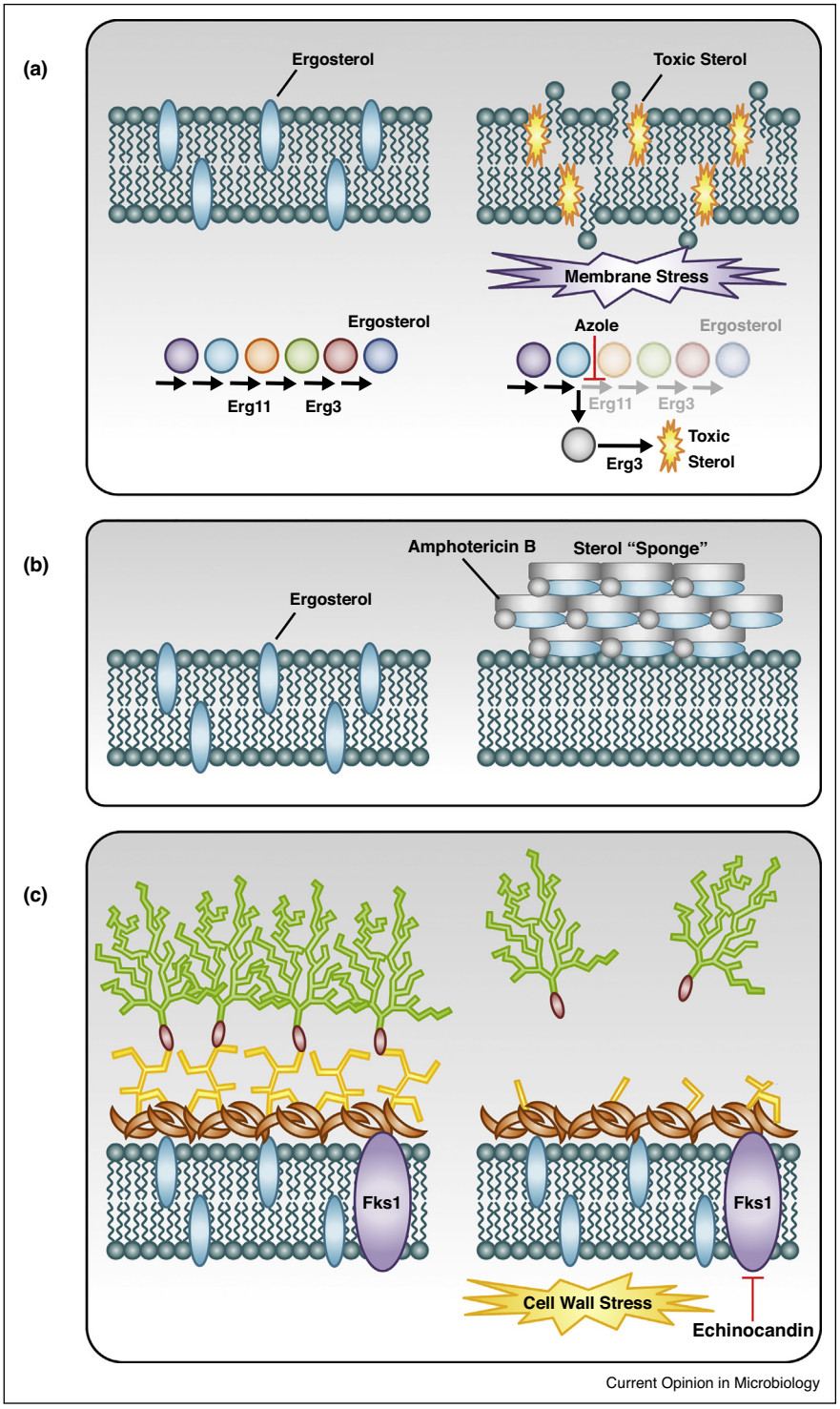
Fungal pathogens have a profound impact on global human health, food security, and biodiversity. Advances in modern medicine have improved the treatment of diverse human diseases and restricted infectious disease outbreaks, thereby extending human lifespan. As a consequence, opportunistic fungal pathogens have emerged as a leading cause of human mortality, particularly in individuals with underlying health conditions or undergoing immunosuppressive treatments, with attributable mortalities estimated at ~1.5 million per year [1]. The predominant causative agents include species of *Candida*, *Aspergillus* and *Cryptococcus* [1]. *Candida albicans* is a leading cause of nosocomial infections, with mortality rates often exceeding ~40% despite treatment [2]. Non-*albicans Candida* (NAC) species are also problematic with drug-resistant isolates becoming increasingly more common [3•]. *Aspergillus fumigatus* is a ubiquitous saprophytic fungus estimated to cause over 200 000 cases of invasive aspergillosis occur each year, with mortality rates often exceeding 50% [1]. Finally, cryptococcosis, caused by species such as *Cryptococcus neoformans* and *Cryptococcus deuterogattii*, affects over one million individuals annually, often resulting in severe central nervous system infections in vulnerable individuals [1]. While cryptococcosis caused by *C. neoformans* is an AIDS-defining illness, *C. deuterogattii* is distinguished by its propensity to cause infections in otherwise healthy hosts [4]. Fungal pathogens not only impact humans directly, they jeopardize food security through mass devastation of crops that feed billions, as well as producing toxins that contaminate food supplies and lead to the development of cancers [5]. In recent years, there has been an unprecedented number of fungal diseases causing extinctions in wild species, with mass mortalities of bats and amphibians threatening biodiversity. Climate change is poised to exacerbate the problem as increasing global temperatures are being accompanied by pests and pathogens moving northward, with fungi leading the way [6].

The impact of fungi on human health is amplified by the fact that only three classes of antifungal drugs are available to treat systemic fungal infections [3•,7•]. These include the azoles that target ergosterol biosynthesis, the echinocandins that inhibit fungal cell wall biosynthesis, and the polyenes that bind to ergosterol in the fungal cell membrane leading to cell lysis (Figure 1) [3•,7•]. Our limited arsenal of antifungals is further threatened by the development of multidrug-resistant strains of fungi and the emergence of intrinsically resistant pathogens. In this review, we highlight mechanisms of antifungal resistance with a focus on fungal pathogens that infect human hosts. We also touch on promising new therapeutic strategies that may be employed in the future to address this global health crisis.

Acquired resistance mechanisms

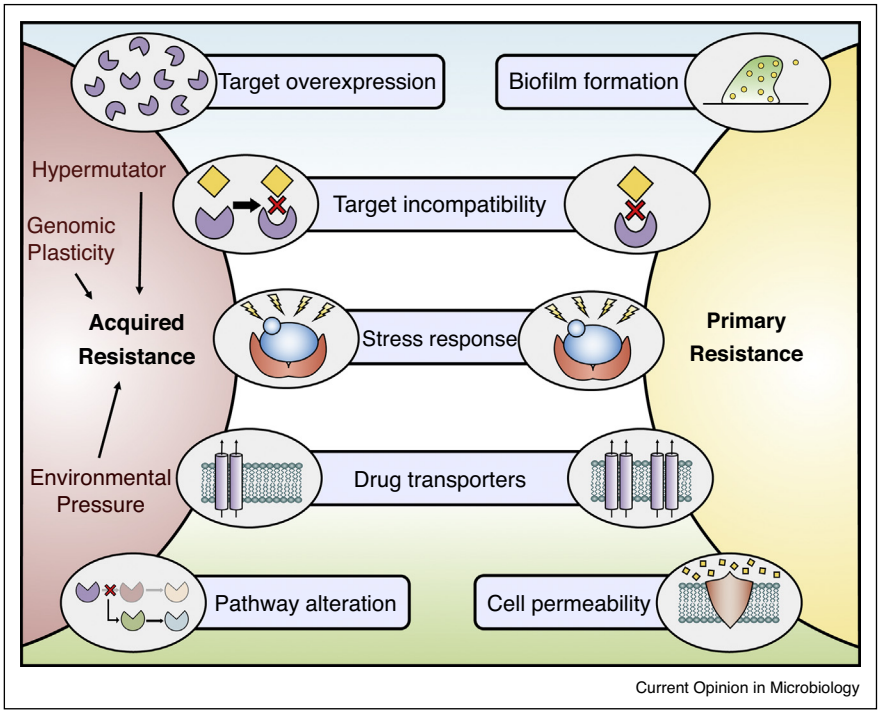
Our frequent and prophylactic use of antifungal agents has led to the development of robust resistance in medically important fungi. Numerous adaptive mechanisms of antifungal drug resistance have been identified, including drug target alteration or overexpression, upregulation of multidrug transporters, and activation of stress responses (Figure 2) [8]. Although resistance to the polyenes remains extremely rare [9], resistance to the azoles and echinocandins is readily documented and their modes of acquired resistance will be elaborated on below.

Figure 1



Antifungal drug mode of action. **(a)** The azoles function by targeting the ergosterol biosynthetic enzyme lanosterol demethylase, encoded by *ERG11* (*C. albicans* and *C. neoformans*) or *cyp51A* and *cyp51B* (*A. fumigatus*), causing a block in the production of ergosterol and the accumulation of toxic sterol intermediates produced by *ERG3.* This toxic sterol induces a severe membrane stress on the cell and ultimately inhibits growth of the fungi. **(b)** The polyenes such as amphotericin B act primarily by forming large, extramembranous aggregates that extract ergosterol from lipid bilayers. **(c)** Fungal cell walls are composed of (1,3)-β-D-glucans covalently linked to (1,6)-β-D-glucans as well as chitin, mannans, and cell wall proteins. The echinocandins act as non-competitive inhibitors of (1,3)-β-D-glucan synthase, encoded by *FKS1* (*C. albicans*, *C. neoformans*, and *A. fumigatus*), thereby causing a loss of cell wall integrity and severe cell wall stress.
Adapted from Cowen LE 2008. Nat. Rev. Micro. 6(3):187–98.

Figure 2



Exploring the relationships between and mechanisms governing intrinsic and acquired resistance. The development of acquired resistance can occur through several mechanisms. Examples include overexpression of the drug target, amino acid substitutions in the drug target that impede drug binding, signaling through stress response pathways, upregulation of efflux pumps, or alterations in cellular pathways. Acquired resistance in fungal pathogens can be accelerated via multiple factors including but not limited to an organisms' genetic plasticity, the existence of hypermutator strains, or environmental pressures that result in strains becoming resistant to agricultural fungicides leading to cross resistance in clinical isolates. Primary resistance is achieved through several mechanisms overlapping with those implicated in acquired resistance including target incompatibility, stress response signaling, and efflux pump overexpression. In addition, the formation of fungal biofilms decreases overall fungal drug susceptibility, and differences in cellular permeability may prevent a drug from reaching its target. The combined effect of these contributing mechanisms leads the selection of increasingly resistant organisms.

Resistance to azoles

Azole antifungals inhibit the ergosterol biosynthetic pathway by targeting the cytochrome P450-dependent enzyme lanosterol 14-α-demethylase, encoded by Erg11 in yeasts, and Cyp51A/Cyp51B in molds. Inhibiting this pathway disrupts the production of ergosterol and results in an accumulation of toxic sterol intermediates that perturb membrane stability and impede fungal growth (Figure 1) [7•]. One of the most prevalent mechanisms of azole resistance involves alteration or overexpression of the drug target gene, *ERG11/cyp51A/cyp51B*, with *Candida* and *Aspergillus* resistant isolates often having amino acid substitutions in regions close to the heme-binding site of the enzyme [10,11]. Furthermore, constitutive overexpression of *ERG11* via gain-of-function mutations in the transcriptional activator Upc2 is commonly found in resistant isolates of *C. albicans* [12]. In other human fungal pathogens, distinct sterol regulatory elements such as the transcription factor Sre1 in *C. neoformans* and SrbA in *A. fumigatus*, have also been implicated in responses to antifungal drugs and virulence [13,14]. Alterations in other components of the ergosterol biosynthetic pathway, such as loss of function of the Δ-5,6-desaturase enzyme Erg3, can also enable azole resistance. *ERG3* mutations lead to the depletion of ergosterol and the accumulation of alternative sterols, often resulting in cross-resistance to azoles and polyenes [15]. *ERG3*-mediated azole resistance intimately depends on key stress response regulators such as the protein phosphatase calcineurin [16], the protein kinase Pkc1 [17], the molecular chaperone Hsp90 [16], and likely other regulators that remain to be identified. In fact, functional genomic screens have identified several genes encompassing diverse cellular processes important for mediating azole tolerance in both *C. albicans* and *C. neoformans* [18,19•,20].

Another common mechanism of acquired azole resistance involves the upregulation of multidrug transporters. The ATP-binding cassette (ABC) transporters Cdr1 and Cdr2, as well as the major facilitator Mdr1 have all been implicated in clinical azole resistance of many *Candida* species [21,22]. Upregulation of Mdr1 has been shown to

simultaneously enable *C. albicans* azole resistance as well as escape from intrinsic host defenses through the efflux of antimicrobial peptides, such as histatin 5 [23]. The expression of *CDR1* and *CDR2* is regulated by the transcription factor Tac1 in *C. albicans*, with *TAC1* alleles harboring gain-of-function mutations readily identified in resistant isolates [24]. Similarly, mutations in the transcription factor gene *MRR1* lead to upregulation of Mdr1 in azole-resistant isolates of *C. albicans* [25]. In *C. neoformans* and *A. fumigatus*, the ABC transporters responsible for azole efflux are Afr1 and AtrF, respectively [26,27].

Genomic alterations that lead to an increased dosage of drug transporters provide an alternative route to enhance drug efflux. Fungal species are capable of remarkable genomic plasticity in response to diverse environmental stresses. Studies examining the genome composition of *C. albicans* azole-resistant isolates identified a duplication of the left arm of chromosome 5 (termed i(5L)) as a common aneuploidy [28]. The formation of i(5L) results in increased dosage of both *ERG11* and *TAC1*, enabling a dual mechanism of azole resistance [28,29]. Recently, other aneuploid lineages of *C. albicans* harboring increased copy numbers of chromosome 3 and chromosome 6 were determined to have reduced susceptibility to azoles [30]. Although the molecular basis remains elusive, these findings further support the notion that genomic diversity promotes stress adaptation and survival in *C. albicans*. Finally, in *C. neoformans*, duplication of chromosome 1, the genomic location of the azole target gene *ERG11*, was discovered as an adaptive mechanism to confer azole resistance [31]. Thus, genomic plasticity appears to be a conserved and central adaptive mechanism (Figure 2).

Given the large-scale deployment of azoles in agriculture, the potential for pathogens that acquired resistance in the field to be transmitted to human hosts is cause for grave concern. *A. fumigatus* with environmentally acquired resistance has been identified in azole-naïve patients without previous azole exposure [32]. Specifically, in a study of 144 soil samples collected from greenhouses in China, 5.8% of the analyzed samples displayed cross-resistance with agro-chemicals and medical azoles [33•]. Intriguingly, exposure to environmental azoles has also been shown to induce cross-resistance to azole antifungals in both *C. neoformans* and *C. gattii*, mostly due to mutations in *ERG11* [34].

Resistance to the echinocandins

The echinocandins are the newest class of antifungal drug released into the clinic. They target the fungal cell wall by inhibiting (1,3)-β-D-glucan synthase, encoded by *FKS1* (and *FKS2* in *Candida glabrata*), inducing a severe cell wall stress and leading to a loss of cell wall integrity (Figure 1) [7•]. These first-line drugs have the advantage of being fungicidal against the majority of *Candida* species, although they are predominantly fungistatic against *A. fumigatus* [7•]. Echinocandin resistance is primarily conferred via amino acid substitutions within highly conserved regions of the Fks subunits of glucan synthase [35]. These hot spot regions are conserved across different *Candida* species, with levels of resistance varying depending on the mutations and expression level of these genes [15]. In *C. glabrata*, *FKS2* expression is calcineurin dependent, therefore resistance conferred by *FKS2* can be reversed upon administration of calcineurin inhibitors such as FK506 [15,36].

Complex cellular circuitry orchestrating responses to cell wall stressors can also confer echinocandin tolerance and resistance. Cell wall integrity signaling mediated via protein kinase C (PKC), the protein phosphatase calcineurin, and the molecular chaperone Hsp90, is vital in enabling echinocandin drug tolerance and compensatory mechanisms such as upregulation of chitin synthesis [17,37,38]. Additionally, a recent study implicated the *C. albicans* transcription factor Cas5 in governing echinocandin tolerance and resistance [39•]. Similar to the azoles where diverse cellular processes modulate susceptibility, diverse genes have been implicated in echinocandin tolerance in fungal pathogens [18,19•,20]. The genes required to respond to different drug classes appear largely distinct and species restrictive, suggesting specificity in the circuitry governing cellular responses to stress.

More recently, strains with acquired resistance to multiple classes of antifungal drugs have become of grave concern. One mechanism by which this occurs is via the emergence of hypermutator lineages. One poignant example of this has been reported in *C. glabrata* where one third of the 1300 isolates analyzed in one study were identified as non-susceptible to both an echinocandin and an azole [40]. This multidrug resistance phenotype was likely attributable to a hypermutator phenotype [40,41••], as approximately 55% of resistant clinical isolates had loss-of-function mutations in the DNA repair gene *MSH2*, accelerating the emergence of multidrug resistance [41••]. Recently, a hypermutator lineage was also identified in *C. deuterogattii* where a mutation in *MSH2* enabled elevated mutation rates [42••]. Interestingly, while hypermutators in bacterial populations often produce offspring that are generally less fit, only modest fitness defects have been observed with hypermutators in fungi [41••,42••]. Further, although in *C. deuterogattii* loss of function of the mismatch repair component *MSH2* does not appear to directly impact virulence, it may provide a means to promote loss of virulence over time as mutations accumulate in critical pathways [42••]. Although relatively little is known about hypermutators in eukaryotes, these examples suggest that this may be a prevalent adaptive strategy in eukaryotic pathogens (Figure 2).

The increasing prevalence of primary resistance

In addition to the ability to acquire resistance through mutations or genomic alterations, fungal species that are inherently resistant to antifungals have become a growing problem in medicine and agriculture. Inherent resistance, or primary resistance, is used to describe species in which all known isolates possess an innate resistance to an antifungal (Figure 2). A classic example of this is the resistance of *Cryptococcus* species to echinocandins. This has remained enigmatic as *C. neoformans* not only possesses a β-glucan synthase encoded by *FKS1*, but echinocandins inhibit the enzyme effectively *in vitro* [43]. Recently, a screen of over 7000 *C. neoformans* mutants identified mutations in *CDC50* that rendered the strain sensitive to echinocandins [44]. *CDC50* encodes the β-subunit of a lipid flippase involved in phospholipid translocation and trafficking, and is required for membrane integrity, stress resistance and virulence [44].

Variation in resistance phenotypes is observed not only between species and among isolates of the same species, but also between distinct growth states for the same strain. Microbial biofilms are surface-associated communities that exhibit remarkable levels of drug resistance relative to their planktonic counterparts [45]. The glucan matrix that surrounds fungal biofilms can act as a physical barrier to impede the bioavailability of antimicrobial compounds. Other factors such as alterations in efflux pump expression, changes in cell membrane and wall composition, and alterations in stress response profiles also contribute to the altered drug resistance profiles of these communities [45].

Finally, the emergence of pathogenic species with elevated resistance to current antifungal drugs is concerning. This is apparent with the increased prevalence of resistant NAC species, such as *Candida tropicalis*, *C. glabrata*, and *Candida parapsilosis*, and with the recent global emergence of *Candida auris* [46]. Since its first isolation in 2009, there have been several clinical outbreaks of *C. auris* suggesting that this pathogen has exceptional adaptive mechanisms to persist in hospital environments that remain to be identified [46]. The most comprehensive assessment of *C. auris* resistance to date found that 93% of isolates have extremely high levels of resistance to the azole fluconazole (≥32 μg/mL) [47]. Here, the distinction between intrinsic and acquired resistance becomes less clear, as independently arising mutations suggest an acquired mechanism, however, no sensitive pools of *C. auris*, in which azole treatment would be effective have been identified. This intrinsic resistance could be explained by the combined effect of an expansion of multidrug transporters in the genome and nine amino acid changes in Erg11, which are known to confer significant resistance in *C. albicans* [47,48]. Even more concerning, 4% of *C. auris* isolates were resistant to all three classes of antifungals, leaving no therapies for these formidable infections [47].

Conclusion: outlook for future antifungal development

A major obstacle in antifungal discovery is identifying essential targets in fungi that can be specifically engaged by molecules that lack host toxicity. A promising strategy to expand the drug target space is to explore combination therapy. This approach is fundamental to the treatment of AIDS, malaria, and tuberculosis and provides multiple advantages, including reducing the rate of resistance, increasing potency, lowering host toxicity and broadening the therapeutic range [7•]. Drug combinations can also be employed to target resistance mechanisms themselves, particularly those involved in drug tolerance. For example, *C. glabrata* azole resistance is frequently achieved through substitutions in the transcription factor Pdr1, resulting in upregulation of efflux. By inhibiting the interaction of the Pdr1 activation domain and the mediator Gal11A in resistant *C. glabrata* isolates, the compound iKIX1 can restore azole sensitivity and reduce fungal proliferation in murine systemic infection models [49••]. Targeting stress response regulators such as the molecular chaperone Hsp90 has also been shown to have vast therapeutic potential to increase the efficacy of both azoles and echinocandins while impairing the emergence of resistance [16,38,50]. Advances in high throughput screening and chemical genomic approaches in the model yeast *Saccharomyces cerevisiae*, as well as the fungal pathogens *C. albicans* and *C. neoformans* have enabled powerful strategies to identify new chemical matter and targets for antifungal drug development [51–53]. Although antifungal resistance continues to emerge, recent advances suggest that an expansion of the current arsenal of antifungal treatments is on the horizon.

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