

**REVIEW**

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# Elucidating drug resistance in human fungal pathogens

Jinglin Lucy Xie<sup>1,‡</sup>, Elizabeth J Polvi<sup>1,‡</sup>, Tanvi Shekhar-Guturja<sup>1,‡</sup>  
& Leah E Cowen<sup>\*1</sup>

**ABSTRACT:** Fungal pathogens cause life-threatening infections in immunocompetent and immunocompromised individuals. Millions of people die each year due to fungal infections, comparable to the mortality attributable to tuberculosis or malaria. The three most prevalent fungal pathogens are *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Fungi are eukaryotes like their human host, making it challenging to identify fungal-specific therapeutics. There is a limited repertoire of antifungals in clinical use, and drug resistance and host toxicity compromise the clinical utility. The three classes of antifungals for treatment of invasive infections are the polyenes, azoles and echinocandins. Understanding mechanisms of resistance to these antifungals has been accelerated by global and targeted approaches, which have revealed that antifungal drug resistance is a complex phenomenon involving multiple mechanisms. Development of novel strategies to block the emergence of drug resistance and render resistant pathogens responsive to antifungals will be critical to treating life-threatening fungal infections.

The devastating effects of fungal infections on human health worldwide remain largely unappreciated. Pathogenic fungi infect billions of people every year, with over 1.5 million of these infections resulting in death [1]. In fact, fungi kill as many people annually as malaria or tuberculosis [1]. Fungi are generally opportunistic pathogens, preying on individuals with compromised immune systems including those with HIV/AIDS, those receiving immunosuppressive drugs for organ transplantation, and those undergoing cancer treatment [2]. As the number of severely immunocompromised individuals increases, so does the incidence of invasive fungal infections. In recent years, the occurrence of systemic fungal infections has increased by 207% [2].

Among the most deadly fungal pathogens are *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Figure 1), with *C. albicans* reigning as the most prevalent invasive fungal pathogen of humans [2]. *C. albicans* is a natural member of the human microbiota; however, it is capable of causing severe systemic infections in immunocompromised individuals, with mortality rates that approach 40% [3]. Other *Candida* species, such as *Candida glabrata* and *Candida parapsilosis*, are also common causes of invasive mycoses [2]. *C. neoformans* is an opportunistic pathogen, with immunocompromised individuals acquiring infections from environmental sources [4]. *C. neoformans* is the third most common cause of CNS complications in AIDS patients [4]. Over 600,000 deaths are attributable to the 1 million new cases of cryptococcal meningitis that occur every year [5]. Finally, the filamentous mold *A. fumigatus* is the most common cause of invasive aspergillosis, with mortality rates of 40–90% [6]. Inhalation of *A. fumigatus* conidia can also lead to allergic bronchopulmonary aspergillosis in patients with pulmonary disorders [6].

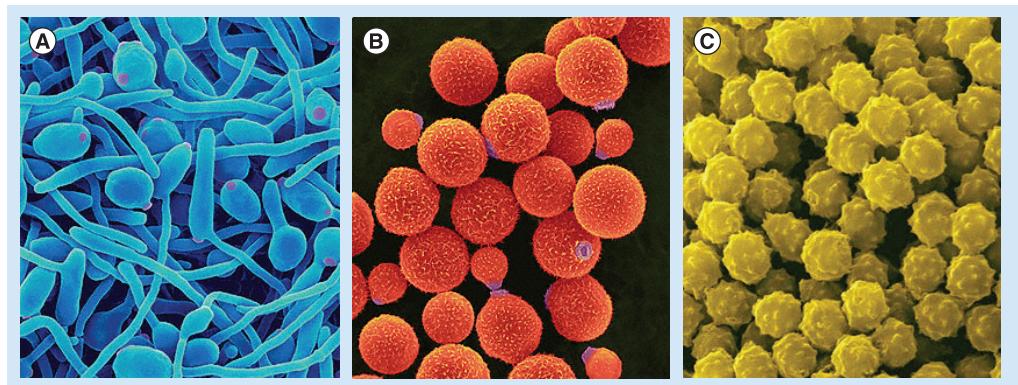
**KEYWORDS**

- *Aspergillus fumigatus*
- azoles • *Candida albicans*
- *Cryptococcus neoformans*
- drug resistance
- echinocandins • polyenes

<sup>1</sup>Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

\*Author for correspondence: Tel.: +1 416 978 4085; Fax: +1 416 978 6885; [leah.cowen@utoronto.ca](mailto:leah.cowen@utoronto.ca)

<sup>‡</sup>Authors contributed equally



**Figure 1.** The three leading fungal pathogens of humans are *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Scanning electron microscopy images of (A) *Candida albicans* yeast and filaments (800 $\times$  magnification), (B) *Cryptococcus neoformans* budding yeast (1200 $\times$  magnification) and (C) *Aspergillus fumigatus* spores (1200 $\times$  magnification). Images copyright Dennis Kunkel Microscopy, Inc. (HI, USA).

Estimated mortality rates for these invasive infections range from 20 to 95%, depending on the pathogen and patient population [1], despite currently available treatment options. Since fungi are eukaryotes like their human hosts, there are a limited number of drug targets that can be exploited to selectively kill the pathogen with minimal host toxicity. As such, new classes of antifungal drugs have not reached the clinic since the late 1990s. Most antifungals in clinical use target the cell membrane sterol ergosterol, or its biosynthesis, or the cell wall linker molecule 1,3- $\beta$ -D-glucan (Figure 2). Resistance to the available classes of antifungals has emerged as a severe problem, with fungal infections becoming increasingly difficult to treat. Recently, the CDC listed azole-resistant *Candida* as a serious threat to human health, at the same threat level as MRSA, causing approximately 46,000 infections annually among hospitalized patients [7]. Fungal species may have intrinsic resistance to a specific antifungal drug or class, or fungi may readily evolve resistance upon exposure to the antifungal drug [8]. Resistance in fungal pathogens can lead to therapeutic failures and poor clinical outcome for patients suffering from life-threatening fungal infections.

This article focuses on the mechanisms of resistance to the most widely deployed classes of antifungals in the most prevalent fungal pathogens of humans, and the approaches that have been exploited to uncover these mechanisms. This will provide a framework for elucidating strategies to combat the emergence of antifungal resistance in these deadly human pathogens.

### Major classes of antifungal drugs

Currently, there are three major classes of antifungals in clinical use for the treatment of fungal infections in humans: the polyenes, the azoles and the echinocandins (Figure 2). The polyenes include amphotericin B and nystatin, and were the first class of antifungal to reach the clinic over 50 years ago [10]. These amphipathic molecules bind to the fungal cell membrane sterol ergosterol, creating pores in the cell membrane that allow intracellular ions to leak out [8]. The mechanism of action of polyenes may be more complex, as they can also inhibit membrane transport proteins in addition to their effects on cellular permeability [11]. Polyenes have a broad spectrum of activity against species of *Candida*, *Cryptococcus* and *Aspergillus*, but suffer from considerable host toxicity problems [3]. The most widely used antifungals are the azoles, including fluconazole, voriconazole and posaconazole. Azoles function by inhibiting lanosterol demethylase, an enzyme involved in the biosynthesis of ergosterol; this leads to depletion of ergosterol and accumulation of a toxic sterol intermediate, 14 $\alpha$ -methylergosta-8,24(28)-dien-6 $\beta$ ,3 $\alpha$ -diol, thereby disrupting the integrity of fungal cell membranes [3]. The azoles are generally fungistatic against yeasts and fungicidal against molds [3]. The newest class of antifungal to reach the clinic is the echinocandins, including anidulafungin, micafungin and caspofungin. They impair integrity of the fungal cell wall by inhibiting synthesis of a structural polysaccharide, 1,3- $\beta$ -D-glucan [12]. Echinocandins generally have fungicidal activity against *Candida* species

and fungistatic activity against *Aspergillus* species, although they are clinically ineffective against *Cryptococci* [3].

Beyond the three major classes of antifungals, there are a limited number of antifungals in clinical use or in development. Flucytosine is the only antifungal in the pyrimidine class that is approved for clinical use [3]. It targets DNA synthesis, but its antifungal activity is restricted to *Candida* species and *C. neoformans* [13]. Owing to the rapid emergence of resistance to flucytosine, it is commonly used only in combination with amphotericin B, particularly in the treatment of cryptococcal meningitis [10]. Antifungals in development include sordarins and nikkomycin Z. Sordarins inhibit fungal elongation factor 2, thereby blocking protein biosynthesis, with the derivative FR290581 in clinical development [14]. Nikkomycin Z inhibits cell wall biosynthesis by targeting chitin synthases, with Phase I clinical trials underway [14].

The prevalence of drug resistance varies by species and class of antifungal. Perhaps the most surprising, is that polyene resistance remains rare, despite a long history of clinical use. This is likely due to the finding that mutations conferring polyene resistance in *C. albicans* are associated with fitness trade-offs, including hypersensitivity to oxidative stress, febrile temperatures and neutrophil killing, as well as causing impaired morphogenesis and tissue penetration [15]. By contrast, resistance to azoles is far more prevalent, perhaps as a consequence of their fungistatic activity, which exerts strong selection for resistance on fungal populations that survive drug exposure. There are an estimated 3400 cases of infections caused by azole-resistant *Candida* species in healthcare-associated infections in the USA alone, resulting in 220 deaths annually [7]. Species of *Candida* that are intrinsically resistant to azoles, such as *C. glabrata*, are increasingly prevalent in the clinic worldwide [16,17], and azole resistance in *C. neoformans* remains high in Africa [18]. *A. fumigatus* is intrinsically resistant to fluconazole, and an increase in resistance to other azoles has been observed in patients treated with azoles and even those who were not [19], with the agricultural use of azoles providing a potential reservoir for the dissemination of resistant strains [20]. As with azoles, species of *Candida* with elevated resistance to echinocandins are increasing in prevalence in clinics where echinocandins are utilized, as is the case with *C. parapsilosis* [21,22]. Recurrent infections

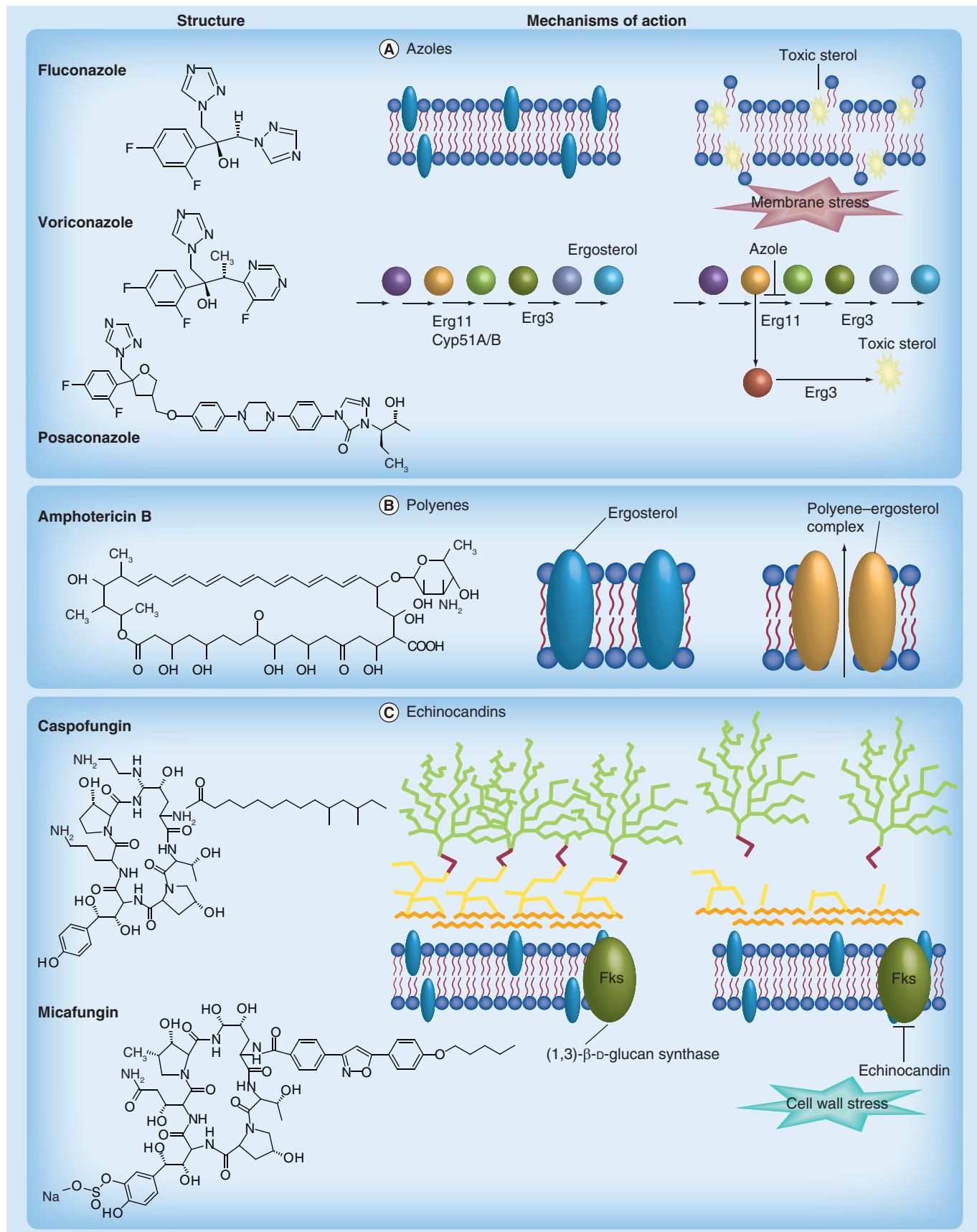
in patients treated with echinocandins underpin concern regarding the emergence of echinocandin resistance in *Candida* and *Aspergillus* species [23,24], while *Cryptococcus* is intrinsically resistant to echinocandins. Given the limited number of antifungal drug classes and the prevalence of cross-resistance to all drugs in a class, antifungal drug resistance should not be overlooked.

### Approaches to elucidating mechanisms of drug resistance

Drug resistance is a complex trait involving multiple mechanisms. Our current understanding of mechanisms controlling antifungal drug resistance has been informed through complementary approaches to determine differences between resistant isolates and their susceptible counterparts. Much of the early work on drug resistance focused on the model yeast *Saccharomyces cerevisiae* and the pathogenic yeast *C. albicans*, given the availability of genetic tools. Here, we highlight approaches to elucidate drug resistance in pathogenic fungi (Figure 3).

- **Genetic screens to identify resistance determinants**

Forward and reverse genetics provide powerful approaches for analyzing diverse traits, including drug resistance. The basic principle underlying both approaches is to explore the genetic basis of a mutant phenotype. Forward genetics was one of the earliest approaches used to identify drug-resistance mutations, based on selection of drug-resistant mutants. Resistance mutations are often further characterized in *S. cerevisiae* by classical genetic means such as genetic crosses, complementation and dominance tests. Notably, genes encoding the drug target for rapamycin and the echinocandins were both identified based on analysis of spontaneous-resistant mutants of *S. cerevisiae* [25,26]. Identification of recessive-resistance mutations in *S. cerevisiae* can be facilitated by expression of barcoded plasmids with each wild-type gene to identify which gene complements the resistance phenotype by pooled analysis of fitness [27]. Functional genomics approaches have also been developed to identify drug-resistance mutations based on simultaneous screening of high complexity, randomly mutagenized libraries of approximately 90% of *S. cerevisiae* genes [28]. Recent advances in whole-genome sequencing technologies coupled with forward genetics offer exciting opportunities to study resistance mechanisms that evolve



**Figure 2. Mechanism of action of the major classes of antifungal drugs (facing page).** (A) Azoles inhibit the ergosterol biosynthesis enzyme lanosterol demethylase encoded by *ERG11* in *Candida albicans* and *Cryptococcus neoformans* or by *cyp51A* and *cyp51B* in *Aspergillus fumigatus*; azoles inhibit ergosterol production and cause accumulation of a toxic sterol generated by Erg3, leading to cell membrane stress. The colored circles represent intermediates in ergosterol biosynthesis. (B) Polyenes bind to ergosterol creating drug–lipid complexes that intercalate into the fungal cell membrane forming a channel that spans the membrane; polyenes cause leakage of cellular ions, destroying the proton gradient and causing osmotic cellular lysis. (C) Echinocandins inhibit (1,3)- $\beta$ -D-glucan synthase, which is encoded by *FKS1* in *C. albicans*, *C. neoformans* and *A. fumigatus* and by *FKS1* and *FKS2* in *Candida glabrata* and *Saccharomyces cerevisiae*; (1,3)- $\beta$ -D-glucan is a key structural component of the fungal cell wall, and inhibition of its synthesis causes loss of cell wall integrity and cell wall stress.

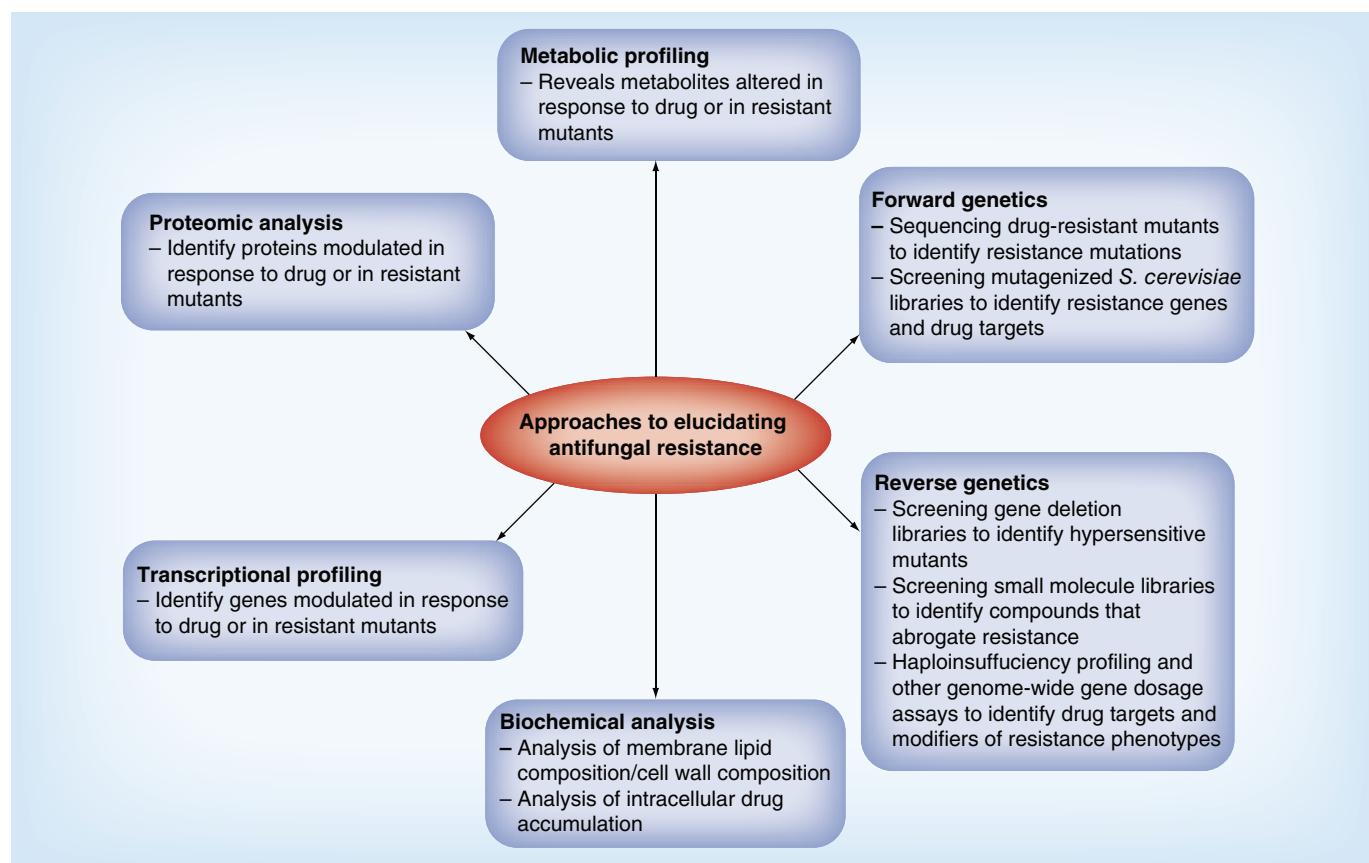
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*in vitro* and *in vivo*, facilitating the identification of clinically relevant resistance mutations [29,30].

Over the last decade, the availability of diverse fungal genome sequences has enabled the development of reverse genetic approaches. Reverse genetics involves targeting genes of interest for disruption and assessing the phenotypic consequences. The construction of mutant libraries has enabled large-scale systematic screening. Mutants have been screened for drug-susceptibility phenotypes, leading to the identification of genes involved in resistance to caspofungin [31], amphotericin B [28] and fluconazole [32].

A complementary strategy involves libraries of pharmacological compounds that can be screened to identify molecules that abrogate drug resistance. For example, screening the LOPAC<sup>1280</sup> Navigator library revealed that inhibition of protein kinase C (PKC) abrogates azole resistance of *C. albicans*, and further genetic analysis implicated the Pkc1-dependent cell wall integrity pathway in modulating resistance to drug-induced membrane stress [33]. Genetic approaches such as haploinsufficiency profiling can reveal targets of drugs that modulate drug resistance, based on the principle that reducing



**Figure 3. Overview of approaches to elucidating antifungal drug resistance.**

dosage of the drug target confers hypersensitivity to that drug [34]. Integrating additional genome-wide gene dosage assays provides enhanced power to identify drug targets and modulators of resistance phenotypes [35].

- **Biochemical analysis of resistance mechanisms**

Discovery of a drug target and mode of action paves the way for biochemical analyses of drug action and cellular responses. For example, polyenes and azoles target ergosterol or its biosynthesis. Analysis of the membrane lipid composition of *C. albicans* mutants that are resistant to both azoles and polyenes implicated the absence of ergosterol and accumulation of a methylated sterol as a cause for resistance [36]. Analysis of the intracellular accumulation of azole demonstrated reduced azole accumulation in azole-resistant isolates compared to their susceptible counterparts [37]; gene expression analysis later demonstrated that overexpression of drug efflux transporters is a key mechanism of azole resistance [38]. Furthermore, analysis of drug–target interaction in an azole-resistant mutant implicated decreased interaction between azole and Erg11 as a cause of resistance [39]. In the context of echinocandins, which inhibit cell wall synthesis, analysis of cell wall composition in response to echinocandin exposure revealed that increased chitin levels confers resistance to echinocandins [40,41].

- **Global analysis of transcripts, proteins & lipids in drug response & resistance**

Technological advances have provided powerful tools for systems level analyses, enabling resistance circuitry to be dissected with unprecedented resolution. Gene expression analysis in *S. cerevisiae* using microarrays revealed rapid upregulation of cell wall genes in response to caspofungin [42]. Proteomic analysis in *C. albicans* using 2D gel electrophoresis coupled to mass spectrometry identified proteins upregulated in response to ketoconazole, amphotericin B and caspofungin, and those that are part of the adaptive response to all three antifungals [43]. Comparative lipidomics revealed quantitative and qualitative changes in lipid classes in azole-resistant *C. albicans* clinical isolates relative to their susceptible counterparts [44]. Analysis of metabolite profiles of *Candida* strains revealed changes in key metabolic pathways in response to caspofungin, amphotericin B and voriconazole [45]. Integration of transcriptional,

proteomic, lipidomic and metabolomic data is poised to reveal systems level regulatory networks controlling drug response and resistance [46,47]. Elucidating drug resistance circuitry is important not only for identifying drug targets and deciphering drugs' mode of action, but it will ultimately guide the development of strategies to prevent the emergence of drug resistance and improve efficacy of existing antifungal drugs.

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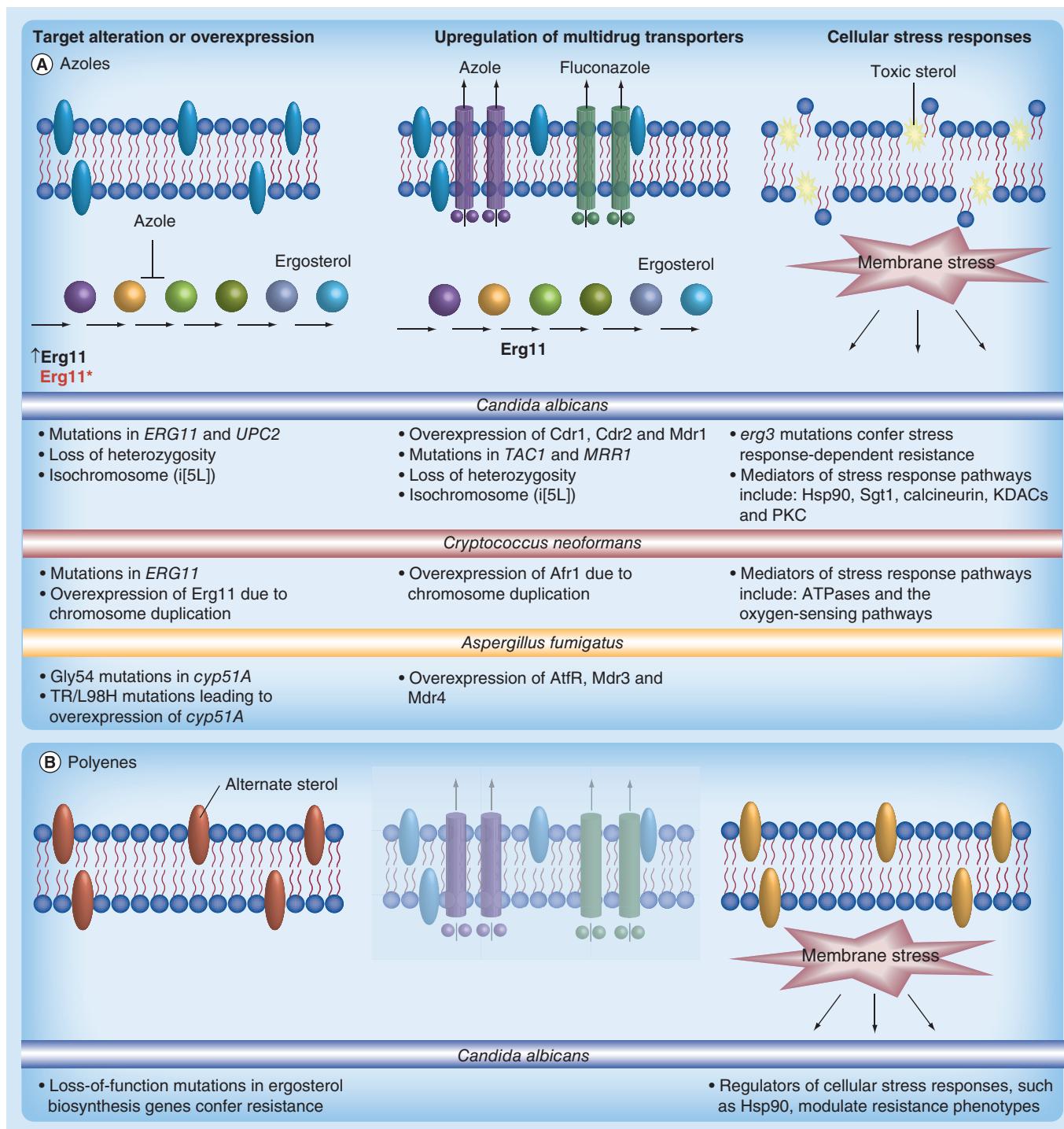
## Mechanisms of drug resistance

- **Drug target alteration**

### Azoles

Alteration of the azole target is a prevalent resistance mechanism in *C. albicans*. The target ergosterol biosynthetic enzyme lanosterol demethylase, encoded by *ERG11*, contributes to resistance when amino acid substitutions reduce azole binding or when overexpression reduces the drug–target dosage relationship (**Figure 4**). Mutations that alter the affinity of Erg11 for azole are clustered in three hot spot regions [48], and many different mutations that confer resistance have been identified [8,49,50]. Ligand-binding studies revealed reduced binding of fluconazole to lanosterol demethylase, with a substitution corresponding to that found in a resistant clinical isolate [49]. Most recently, seven novel *ERG11* mutations were identified in azole-resistant clinical isolates [50]. Mapping the mutations to a 3D model of azole docking at the Erg11 active site [51] revealed their location to be at the substrate entry channel and near the heme-binding site in the active center [50]. Increased *ERG11* expression alone can also confer reduced azole susceptibility *in vitro* [52]; however, in clinical isolates, this is rarely the sole resistance determinant [53]. Overproduction of Erg11 can result from increased copy number of *ERG11* or from substitutions in the transcription factor Upc2, which regulates *ERG11* expression [54]. A recent analysis of 63 fluconazole-resistant clinical isolates revealed that almost half contained a mutation in *UPC2*, the majority of which resulted in increased ergosterol content [55].

As with *C. albicans*, mutations in the drug target gene, *ERG11*, have also been identified in azole-resistant clinical isolates of *C. neoformans* (**Figure 4**) [56]. Sequencing of a fluconazole-resistant isolate recovered from an AIDS patient with cryptococcal meningitis revealed a G484S substitution, which corresponds to the G464S substitution, commonly identified in azole-resistant *C. albicans* strains [56].



**Figure 4. Mechanisms of resistance to azoles and polyenes.** (A) Resistance to azoles can arise through multiple mechanisms including overexpression or alteration of the drug target, overexpression of multidrug transporters, or cellular alterations that mitigate drug toxicity or enable responses to drug-induced stress. The colored circles represent intermediates in ergosterol biosynthesis. (B) Resistance to polyenes primarily involves depletion of the target ergosterol due to loss-of-function mutations in ergosterol biosynthesis genes. Bullet points describe resistance mechanisms for *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Dimmed images indicate those mechanisms that do not play a key role in resistance to the specific drug class. Adapted with permission from [9] © Macmillan Publishers Ltd: Nature Publishing Group, *Nature Reviews Microbiology* (2008). For color images please see [www.futuremedicine.com/doi/full/10.2217/fmb.14.18](http://www.futuremedicine.com/doi/full/10.2217/fmb.14.18)

Consistent with the other species, target alteration is a central mechanism of azole resistance in *Aspergillus* (**Figure 4**). In *A. fumigatus* the azole target enzyme lanosterol demethylase is encoded by *cyp51A*. One of the most prevalent substitutions is at glycine 54; it was first reported in clinical *A. fumigatus* isolates resistant to posaconazole [57], and then in those resistant to itraconazole [58]. Mapping this mutation to a model of azole docking to *A. fumigatus* lanosterol demethylase revealed its location in the pocket in which the side chains of posaconazole and itraconazole bind [59]. A multiplex assay using molecular beacons for mutant Gly54 alleles revealed that almost half of analyzed azole-resistant laboratory and clinical isolates contained this mutation [60]. The other most prevalent *cyp51A* alteration associated with azole resistance in *Aspergillus* involves two distinct mutations: a leucine-to-histidine substitution at position 98 (L98H), as well as a tandem repeat of a 34-bp sequence in the *cyp51A* promoter [61]. Both alterations are necessary to confer resistance and, unlike the Gly54 substitution, this combination yields cross-resistance to various azoles [61]. Large-scale analyses indicate that this mechanism of resistance is the most widespread [62,63]. Interestingly, analyses of *Aspergillus* isolates obtained from Dutch patients revealed the L98H/tandem repeat mutation to be the most prevalent azole resistance mechanism [62,64], despite the majority of patients having never been treated with azoles [64]. This mutation is also commonly observed among environmental *Aspergillus* isolates [63], suggesting possible patient colonization from environmental sources.

#### Polyenes

Resistance to polyenes has been documented in clinical *Candida* isolates from patients receiving either polyene or azole treatment [65], and is often attributable to mutations in genes encoding components of the ergosterol biosynthesis pathway, which lead to depletion of the target sterol (**Figure 4**). Genome sequencing of polyene-resistant *C. albicans* and *Candida tropicalis* clinical isolates revealed mutations in *ERG2*, *ERG3* and *ERG11* [15]. Sequencing of polyene-resistant *C. glabrata* isolates from patients treated with amphotericin B identified a missense mutation in *ERG6* [66]. Furthermore, azole-resistant *C. albicans* clinical isolates containing a defective sterol Δ5,6 desaturase (Erg3) are cross-resistant to amphotericin B [67,68]; cross-resistance is due

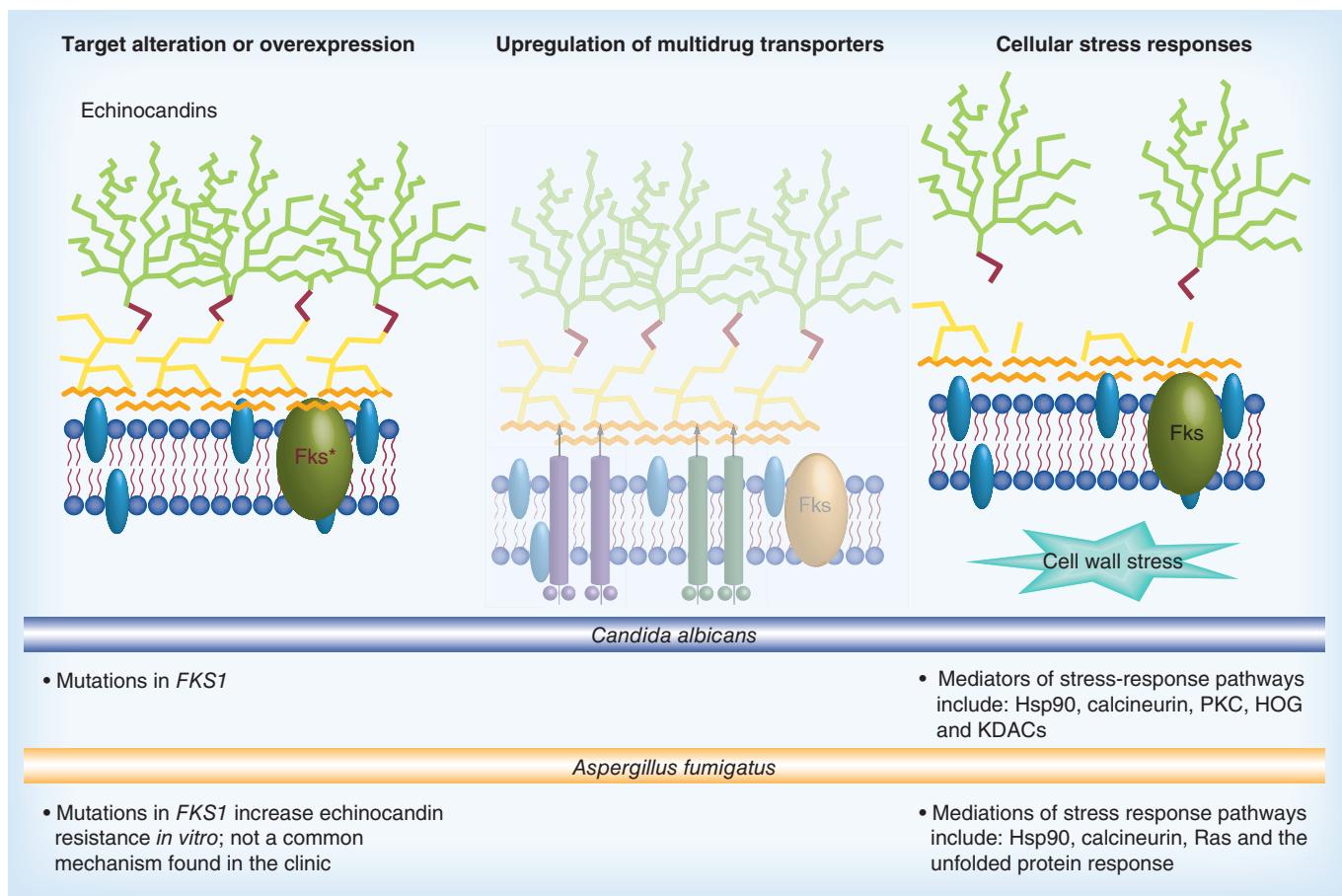
to reduced ergosterol content and accumulation of an alternate sterol [68]. Recently, cross-resistance between azoles and amphotericin B has also been observed in a clinical *C. tropicalis* isolate, which harbored mutations in both *ERG11* and *ERG3* [69].

Amphotericin B, either alone or in combination with other antifungals, is the first line of treatment for cryptococcal infections [70], and *C. neoformans* resistance to this polyene remains fortuitously rare [71]. However, amphotericin B-resistant *C. neoformans* clinical isolates have been documented, with reduced ergosterol content in the cell membrane. For example, amphotericin B resistance of an isolate from an AIDS patient was due to depleted ergosterol as a result of a defective sterol Δ8–7 isomerase [72]. Alterations in other pathways that regulate cell membrane sterol content, such as the high osmolarity glycerol pathway, also influence amphotericin B susceptibility of *C. neoformans* [73].

In contrast to *C. neoformans*, resistance to amphotericin B is common in *Aspergillus* [74], with resistance varying among species. Altered ergosterol content has not been found to contribute to amphotericin B resistance or clinical failure. Instead, the differential susceptibility to amphotericin B observed between *Aspergillus* species may be related to the ability of amphotericin B to function as an oxidizing agent and produce reactive oxygen species. For instance, the increased resistance of *Aspergillus terreus* to amphotericin B compared with *A. fumigatus* may be due to higher endogenous levels of catalase [75].

#### Echinocandins

Alterations in the target of the echinocandins, specifically the catalytic Fks subunits responsible for the synthesis of 1,3-β-D-glucan, is the prevailing cause of acquired echinocandin resistance identified to date (**Figure 5**). Single nucleotide substitutions in two hot spot regions of *FKS1* are often implicated in echinocandin resistance [76]. The first region corresponds to amino acids 641 to 648, with the most frequent substitution being at Ser645 [77]. Analysis of 85 caspofungin-resistant *C. albicans* clinical isolates identified 93% with mutations at Ser645 [77]. The second hot spot was identified in *S. cerevisiae* and includes amino acids 1345 to 1365 [78]. A mutation in this region was identified in an echinocandin-resistant *Candida krusei* isolate [78]. Mutations in the hot spot region 1 of both *FKS1*



**Figure 5. Mechanisms of echinocandin resistance.** Mutations in *FKS1*, which encodes the (1,3)- $\beta$ -D-glucan synthase catalytic subunit, are the most prevalent mechanism of echinocandin resistance. Resistance phenotypes are modulated by cellular stress response pathways. Bullet points describe resistance mechanisms for *Candida albicans* and *Aspergillus fumigatus*. Dimmed images indicate mechanisms that do not play a key role in resistance.

KDAC: Lysine deacetylase; PKC: Protein kinase C.

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and *FKS2* have been identified in bloodstream isolates of *C. glabrata* [79], and a recent global analysis of mutations accompanying the evolution of echinocandin-resistance in *C. glabrata* revealed mutations in *FKS2* and other genes [23]. Polymorphisms in these hot spot regions of other *Candida* species (e.g., *C. parapsilosis*) may contribute to their intrinsic resistance to echinocandins [76]. Finally, a third hot spot region from position 690 to 700 has been identified in *S. cerevisiae* and *C. glabrata* [80]. Substitutions in this region have a distinct impact on resistance to different echinocandins, and may give insight into the mechanism by which echinocandins interact with Fks.

While *FKS1* mutations are frequent causes of reduced echinocandin susceptibility in some *Candida* species, they are not common in other

fungal pathogens. In *A. fumigatus*, the introduction of mutations into *FKS1* can confer resistance to echinocandins [81,82]; however, *FKS1* mutations are rarely identified in clinical isolates (Figure 5). In contrast to *C. albicans* and *A. fumigatus*, *C. neoformans* is intrinsically resistant to echinocandins, despite the essentiality of *FKS1* [83], and the finding that *C. neoformans* Fks1 is sensitive to inhibition by echinocandins *in vitro* [84]. The mechanism by which *C. neoformans* is intrinsically resistant to echinocandins is unknown, but may be attributed to the production of melanin or extracellular degradation of the drug [27].

#### Overexpression of multidrug transporters

One of the predominant mechanisms of azole resistance identified in clinical isolates of diverse

fungal pathogens is the upregulation of multidrug transporters (**Figure 4**). Analysis of azole accumulation in many resistant clinical isolates indicated minimal intracellular accumulation, suggesting that multidrug transporters play an important role in reducing the impact of azoles on fungal cells [3]. Efflux pumps implicated in azole resistance belong to two main classes: the ATP-binding cassette (ABC) and the major facilitator (MF) superfamily of transporters. Both classes comprise of membrane-spanning domains, but utilize different energy sources to actively transport substrates across the cell membrane. ABC transporters have two nucleotide-binding domains and utilize ATP hydrolysis, whereas MF transporters exploit the proton motive force to translocate compounds across the cell membrane [85].

#### • ABC transporters

The ABC transporters have a broad range of substrates and have greater clinical significance compared with MF transporters [85,86]. In *C. albicans*, *CDR1* and *CDR2* play an important role in azole resistance, such that deletion of either gene results in increased susceptibility to azoles [87]. Furthermore, *CDR1* expression is significantly increased (up to tenfold) in many azole-resistant clinical isolates, along with an increase in *CDR2* expression [37,86]. Upregulation of *CDR1* and *CDR2* is also attributed to substitutions in the transcription factor Tac1. Tac1 binds to *cis*-acting drug-responsive elements located in the promoters of these genes, thereby regulating their expression. Constitutive overexpression of *CDR1* and *CDR2* as a result of *TAC1* hyperactive alleles has been observed in *C. albicans* azole-resistant clinical isolates [88]. At least 19 *TAC1* gain-of-function mutations have been described [89–91], and *TAC1* targets have been defined through global transcriptional and occupancy analysis [92]. *CDR1* basal expression is also regulated by the transcription factor Mrr2 [93].

Upregulation of ABC transporters is also a major mechanism of azole resistance in *C. neoformans* and *A. fumigatus*. The *C. neoformans* ABC transporter *AFR1* has been implicated in azole resistance, as elevated cDNA levels were identified in fluconazole-resistant strains. Deletion of *afr1* in a fluconazole-resistant *C. neoformans* strain resulted in susceptibility to fluconazole, implicating *AFR1* as a key resistance determinant [94]. Strains overexpressing *AFR1* are also more virulent than *afr1* mutants in

mouse infection models [95]. In *A. fumigatus*, the ABC transporter *atrF* is upregulated in itraconazole-resistant isolates compared with susceptible counterparts [96].

#### • MF transporters

The MF class of transporters also plays an important role in azole resistance of *C. albicans* and *A. fumigatus*. In *C. albicans*, *MDR1* is upregulated in fluconazole-resistant clinical isolates, and its expression is regulated by the transcription factor Mrr1. Increased levels of *MRR1* accompany *MDR1* upregulation in fluconazole-resistant clinical isolates, as well as in *in vitro*-generated fluconazole-resistant strains [97]. Deletion of *MRR1* from azole-resistant clinical isolates results in loss of *MDR1* expression and multidrug resistance. Furthermore, when introduced into a laboratory strain, *MRR1* alleles from azole-resistant clinical isolates cause upregulation of *MDR1* and multidrug resistance [98]. At least 15 *MRR1* gain-of-function mutations that cause constitutive *MDR1* upregulation have been identified [69,91], and analysis of genome-wide occupancy identified 701 binding sites of hyperactive Mrr1, as well as a binding motif [99]. Although *C. neoformans* has an *MDR1* gene, it has yet to be implicated in antifungal drug resistance [100]. *A. fumigatus*, however, has four Mdr-like pumps, Mdr1 to Mdr4. Analysis of *in vitro*-evolved itraconazole-resistant strains of *A. fumigatus* showed that many strains had either constitutive overexpression of *mdr3* and *mdr4*, or elevated induction of expression of these genes upon itraconazole treatment [101]. While multidrug transporters provide an important mechanism of azole resistance in many fungal pathogens, multiple resistance mechanisms often interact to enable pathogens to survive the cell membrane stress upon azole exposure [3].

In contrast to the azoles, multidrug transporters have negligible impact on resistance to polyenes or echinocandins. This may reflect the location of the targets of these drugs at the cell surface or may reflect substrate specificity of the transporters.

#### Genomic alterations

A striking mechanism by which fungal pathogens can adapt to drug-induced stress is by genomic alterations, particularly in the context of azoles [102]. The genomic plasticity includes chromosomal rearrangements and the formation of isochromosomes, loss of heterozygosity

(LOH) and aneuploidies [102]. In *C. albicans*, aneuploidies are commonly found in fluconazole-resistant clinical isolates and laboratory strains [103]. In particular, chromosome 5 alterations are a frequent cause of azole resistance. The genes encoding both Tac1, the transcription factor responsible for upregulation of ABC transporters, and Erg11, the target of the azoles, are located on the left arm of chromosome 5 [104]. Azole resistance can arise by the acquisition of a *TAC1* hyperactive allele followed by LOH events that renders the hyperactive allele homozygous [104]. Another common chromosome 5 alteration is the formation of an isochromosome (i[5L]), an abnormal chromosome in which two copies of the left arm of chromosome 5 flank the centromere. i(5L) results in an increase in copy number of *TAC1* and *ERG11* [103,104]. Alterations of chromosome 3 can also result in azole resistance. Gain-of-function mutations in *MRR1*, encoding the transcription factor responsible for *MDR1* regulation, followed by LOH due to either mitotic recombination or concerted loss and duplication of chromosome 3, can decrease azole susceptibility [97]. Aneuploidies, and i(5L) in particular, can become fixed in the population and confer increased fitness in both the presence and absence of antifungal stress [105].

As with *C. albicans*, aneuploidies can result in decreased azole susceptibility in *C. neoformans*. In particular, disomes of chromosome 1 correlate with increased resistance to fluconazole [106]. This chromosome contains both the *ERG11* target gene and *AFR1*, which encodes the main *C. neoformans* azole transporter [106]. Recently, chromosome 1 aneuploidies have been identified in azole-resistant *C. neoformans* isolated from the brains of mice. The second most commonly observed disomy in *C. neoformans* is that of chromosome 4 [107]. Located on this chromosome are genes encoding proteins involved in maintaining the endoplasmic reticulum structure and function, namely *SEY1*, *GCS2* and *GLO3*, suggesting a role for the endoplasmic reticulum in promoting formation of specific disomes in response to azoles [107]. In contrast to the *C. albicans* i(5L), which becomes fixed in populations [105], removal of azole stress leads to loss of the *C. neoformans* disomic chromosomes and azole resistance.

Unlike with the azoles, genomic rearrangements have not been documented as having a major impact on polyene or echinocandin resistance. However, analysis of an echinocandin-resistant clinical isolate revealed that high-level

resistance was caused by a spontaneous mutation in *FKS1* followed by LOH [108]. The relative rarity of such events may suggest that azoles specifically promote genomic instability, or that distinct selective constraints minimize the occurrence of genomic alterations in response to stress induced by polyenes or echinocandins.

#### Modulation of cellular stress responses

Adaptation to the cellular stress imposed by antifungal drugs is often contingent upon cellular stress response circuitry. Like all unfavorable environmental conditions, exposure to antifungals activates stress response pathways that confer immediate cellular protection and promote survival. One of the key regulators of cellular stress responses is Hsp90, which stabilizes diverse regulators of cellular signaling [109]. In the context of azoles, Hsp90 enables basal tolerance and resistance acquired by diverse mutations in *C. albicans* and *S. cerevisiae* [110,111]. Hsp90 enables crucial cellular stress responses by stabilizing downstream client proteins. Key client proteins for azole resistance are the protein phosphatase calcineurin and the terminal MAPK of the cell wall integrity pathway, Mkc1 [33,110,112]. Regulators of Hsp90 function, such as the Hsp90 co-chaperone Sgt1, also have a profound impact on azole and echinocandin resistance [113]. Furthermore, lysine deacetylases regulate Hsp90 function and thereby azole resistance in *S. cerevisiae*, and inhibition of lysine deacetylases recapitulates inhibition of Hsp90 in terms of reducing azole resistance in *C. albicans* [114]. In the context of echinocandins, Hsp90 enables basal tolerance and resistance of *C. albicans*, *C. glabrata* and *A. fumigatus* [23,30,110,111]. Hsp90 governs cellular responses to echinocandin-induced cell wall stress through the same client proteins that mediate responses to cell membrane stress induced by azoles [30,33]. Compromising Hsp90 function enhances antifungal activity in multiple models of fungal pathogenesis [30,111], suggesting profound therapeutic benefits of combination therapy.

Additional cellular factors and pathways are important for alleviating drug-induced cellular stress. In the context of azoles, resistance can arise by a loss-of-function mutation in *ERG3*, which encodes one of the ergosterol biosynthetic enzymes in *C. albicans* and *S. cerevisiae* [9]. Erg3 loss of function blocks the production of a toxic sterol that normally accumulates upon inhibition of Erg11 by azoles (**Figure 4**). This

resistance mechanism enables cellular growth with an alternative sterol membrane composition, although azole resistance is contingent upon Hsp90-mediated cellular stress responses [110]. Mutations in *ERG3* are identified in clinical isolates of *C. albicans* that are resistant to azoles [115]. Although *erg3* mutations are sufficient to confer azole resistance *in vitro* they are not *in vivo*, suggesting that cellular stress responses may be compromised in the host [116]. As with azole resistance, polyene resistance acquired by mutations in ergosterol biosynthesis genes also depends on Hsp90 [15]. Notably, these mutants have an elevated requirement for Hsp90 to enable survival even in the absence of polyene, suggesting that cellular stress responses are also crucial for alleviating the internal stress exerted by the resistance mutations [15]. In the context of echinocandins, elevated synthesis of cell wall chitin is an important adaptive mechanism enabling cells to survive the compromised cell wall integrity upon echinocandin exposure [40]. In *C. albicans*, exposure to echinocandins upregulates chitin production via PKC, high osmolarity glycerol and calcineurin signalling, leading to reduced echinocandin efficacy [40,117,118]. Echinocandin resistance due to *FKS1* mutations is associated with constitutive upregulation of cell wall chitin [119]. The PKC cell wall integrity circuitry is also crucial for surviving cell wall stress induced by echinocandins [42] and cell membrane stress induced by azoles [33], emphasizing the central role of cellular stress response circuitry in drug resistance.

#### Cell membrane alterations

Changes in the cell membrane can alter permeability to antifungal drugs or the function of multidrug transporters, or can have more complex effects on drug susceptibility. An important transcription factor implicated in azole resistance of *C. albicans* is Upc2, a key regulator of ergosterol biosynthesis genes. Upc2 binds to *cis*-acting sterol response elements in the promoters of its target genes [120]. *UPC2* is induced upon exposure to azoles, sterol depletion or anaerobic growth conditions, which leads to the upregulation of genes involved in ergosterol biosynthesis [120]. Furthermore, deletion of *upc2* results in reduced ergosterol content and azole hypersusceptibility [54], while gain-of-function mutations in *UPC2* confer azole resistance in clinical isolates [55,97]. Consequently, targeting Upc2 has emerged as a novel strategy to abrogate

azole resistance [121]. Transcriptional regulators of ergosterol biosynthesis genes have also been implicated in azole resistance of other fungal pathogens, as with sterol regulatory element binding protein of *A. fumigatus* [122] and *C. neoformans* [123]. On a broader scale, global changes in the *Candida* lipidome can accompany the emergence of azole resistance [44], and changes in membrane lipids and their fluidity can have profound impacts on azole resistance [124]. Alterations in sphingolipid biosynthesis can also modulate echinocandin resistance [125], emphasizing the importance of the cell membrane in modulating responses to antifungal drugs.

#### Mitochondrial defects

Impaired mitochondrial function has been specifically linked to azole resistance. In *S. cerevisiae* and *C. glabrata*, mutants with loss of mitochondrial function are intrinsically resistant to azoles due to overexpression of transcriptional activators of multidrug transporters [126]. In *C. albicans*, loss of mitochondrial function is not tolerated; however, pharmacological inhibition of mitochondrial complexes enhances azole susceptibility [127]. Mitochondrial defects have not been associated with echinocandin resistance, perhaps because drug transporters have little impact on resistance to this antifungal class. A more detailed discussion of the relationship between mitochondrial function and drug resistance can be found in the following review [128].

#### Biofilms

Biofilms are a pervasive cellular state associated with drug resistance of diverse fungal pathogens. These complex 3D communities form on indwelling medical devices and catheters, and their intrinsic drug resistance often requires surgical removal of the infected device [129]. Azole resistance of *Candida* biofilms is due to multiple factors including an exopolymeric matrix composed of carbohydrates, proteins and nucleic acids that can sequester antifungal drugs, as well as the action of multidrug transporters [130,131]. Complex cellular circuitry regulates biofilm matrix production and drug resistance including PKC cell wall integrity pathway components, alcohol dehydrogenases, glucoamylases, Hsp90 and calcineurin [132–135]. The glucan matrix also sequesters echinocandins, and inhibition of matrix production enhances biofilm susceptibility [136]. Notably, treatment of *C. albicans*

biofilms with fluconazole prior to caspofungin reduces caspofungin efficacy in a manner that is contingent upon Hsp90 and calcineurin [137]. As with *Candida* biofilms, drug resistance of *Aspergillus* biofilms is controlled by matrix, efflux pumps and Hsp90, as well as extracellular DNA [138,139]. A more comprehensive exploration of mechanisms of drug resistance in fungal biofilms is provided in the following review [140].

### Prospects for overcoming drug resistance in the clinic

The myriad mechanisms of drug resistance that have been discovered in recent years provide exciting new targets for antifungal drug development. With the increasing frequency of invasive fungal infections and the limited repertoire of antifungal drugs, developing novel therapeutic strategies to treat life-threatening fungal infections is more important than ever before. Combination therapy provides an attractive option as it can enhance the potency of current antifungals, minimize the dosage of antifungals required compared with single-agent therapies and prevent the emergence of drug resistance in pathogen populations [141]. Drug combinations are fundamental to the treatment of HIV [142], malaria [143] and tuberculosis [144], but have been less well explored for fungal pathogens, with the exception of the combination of flucytosine and amphotericin B for the treatment of cryptococcal meningitis [145]. Effective drug combinations can exploit targeting different stages of the same biochemical pathway such as allylamines and azoles, or targeting different pathways such as biosynthesis of cell wall (echinocandins) and cell membrane (polyenes) components [145]. Targeting cellular regulators important for antifungal drug resistance, such as Hsp90, calcineurin, lysine deacetylases (KDACs) or lysine acetyltransferases, holds great promise for combination therapeutic strategies to improve clinical outcome [111,146]. As next-generation antifungals with enhanced efficacy and lower toxicity are developed, it will be important to examine their utility *in vitro* and in animal models, with the ultimate goal of performing randomized clinical trials in immunocompromised patients to evaluate the clinical utility.

One of the greatest hurdles in the development of new antifungal agents for combination or single-agent therapies is the capacity to selectively target the fungal pathogen to minimize host toxicity. Many key cellular regulators that

have emerged as promising antifungal targets are highly conserved in the human host, as is the case with Hsp90 and calcineurin. Successful exploitation of these targets will likely require the development of fungal selective agents. Calcineurin inhibitors are most broadly utilized as immunosuppressants, necessitating the development of nonimmunosuppressive analogs or fungal selective agents for antifungal therapy [147,148]. Structural divergence of candidate targets between the host and pathogen can be exploited to develop species-specific inhibitors. This concept has begun to be explored with Hsp90 in the context of the protozoan parasite *Typanosoma brucei*, where inhibitors that interact with the parasite protein more strongly than the human counterpart can be rationalized in terms of subtle structural differences [149]. Furthermore, structural analysis of the human and *C. neoformans* farnesyltransferase revealed distinct differences in the substrate-binding pocket between the species, which can provide a foundation for modification of existing farnesyltransferase inhibitors to enhance specificity for *C. neoformans* [150]. Structure-based drug design holds tremendous potential for repurposing compounds currently used in the clinic and facilitating the development of novel therapeutics.

### Targeting the host to thwart fungal pathogens

Given the challenges of antifungal drug resistance, an emerging strategy to treat fungal infections exploits host-pathogen interactions, and host factors that are important for eradicating fungal pathogens. For example, one of the key steps in fungal pathogenesis is fungal binding to host cell receptors. Multiple host cell receptors mediate endocytosis of human fungal pathogens, including E-cadherin and EGFR-HER2 on oral epithelial cells for the uptake of *C. albicans*, CDC44 and RHAMM on the brain microvascular endothelial cells for *C. neoformans*, as well as dectin-1 and E-cadherin on the pulmonary epithelial cells for *A. fumigatus* [151]. Consistent with the importance of these receptors for fungal pathogenesis, EGFR and HER2 inhibitors are effective for treating oropharyngeal candidiasis in mouse models [152]. The discovery of a multitude of fungal antigens that stimulate T-cell-mediated immunity and are immunogenic in murine fungal infection models, such as 1,3-β-glucan, enable the development of vaccines that could prevent fungal

infections [153,154]. An additional challenge for vaccine development is that they must retain efficacy in the immunocompromised patients that are most vulnerable to fungal infections. Recent advances in understanding the genetics of host susceptibility to fungal pathogens and antifungal immunity have illuminated new avenues for the development of immunotherapeutics [155–157]. Antifungal drugs may ultimately synergize with immunomodulatory strategies, as antifungals that target the cell wall can enhance immune recognition [158]. Ultimately, integrated approaches to thwart fungal pathogens hold the greatest promise for eradicating infections and minimizing the evolution of resistance in pathogen populations.

### Conclusion

The emergence of resistance to antifungal drugs compromises the efficacy of the limited number of therapeutic strategies available to treat life-threatening fungal infections. Advances in functional genomics, genome sequencing and systems level analyses have allowed drug resistance circuitry to be dissected with unprecedented power. Understanding drug resistance

mechanisms illuminates new targets that can be exploited to minimize the emergence of drug resistance, and to abrogate resistance once it has evolved in pathogen populations. Development of new therapeutic strategies requires targets that can be selectively inhibited in the pathogen to minimize host toxicity. Host factors can be exploited to block entry of fungal cells, or to modulate immunity and enhance the host capacity to prevent fungal infections or promote fungal clearance. The profound impact of fungal pathogens on human health, despite current treatment options, necessitates the development of innovative strategies to improve clinical outcome.

### Future perspective

The pace of discovery of new antifungal resistance mechanisms and novel therapeutic strategies is poised to accelerate in upcoming years based on advances in technology that can be capitalized on by interdisciplinary teams with expertise spanning microbiology, molecular genetics, functional genomics, bioinformatics, biochemistry, structural biology and immunology. Interactions between academia and industry

## EXECUTIVE SUMMARY

### Prevalence of antifungal drug resistance

- There are a limited number of antifungal drugs for the treatment of invasive fungal infections, with the three main classes being azoles, polyenes and echinocandins.
- Intrinsic resistance of fungal pathogens is prevalent and acquired resistance to available antifungals is on the rise.

### Approaches to elucidating mechanisms of drug resistance

- Both forward and reverse genetic approaches offer powerful strategies to identify fungal drug-resistance mechanisms.
- Systems level analysis of transcriptomes, proteomes and metabolomes complement dissection of cell membrane and cell wall changes in response to antifungal drug exposure or in resistant mutants compared with their sensitive counterparts.

### Mechanisms of drug resistance

- Drug target alterations are a prevalent cause of resistance to the three most widely deployed classes of antifungal drugs.
- Overexpression of multidrug transporters is a prominent mechanism of resistance to azoles, and can be achieved through gain-of-function mutations in transcription factors controlling their expression.
- Cellular stress responses such as signaling through Hsp90, calcineurin and protein kinase C modulate crucial responses to drug-induced cellular stress.

### Prospects for overcoming drug resistance in the clinic

- Combination therapy can increase the effectiveness of current antifungals against resistant pathogens and minimize the emergence of drug resistance.
- Immunomodulatory strategies can promote host clearance of fungal infections and minimize the impact of drug resistance.

will be key for translating new discoveries into clinically useful therapeutics. Ultimately, illuminating strategies to cripple fungal pathogens of humans may illuminate new ways in which we can combat fungal pathogens that threaten our food supplies and biodiversity on a global scale.

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