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Antifungal Drug Resistance: Molecular Mechanisms in *Candida albicans* and Beyond

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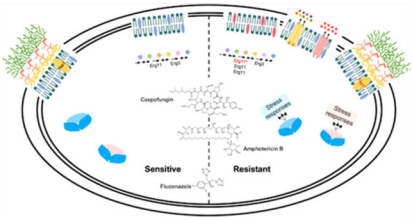
Abstract

Fungal infections are a major contributor to infectious disease-related deaths across the globe. *Candida* species are among the most common causes of invasive mycotic disease, with *Candida albicans* reigning as the leading cause of invasive candidiasis. Given that fungi are eukaryotes like their human host, the number of unique molecular targets that can be exploited for antifungal development remains limited. Currently, there are only three major classes of drugs approved for the treatment of invasive mycoses, and the efficacy of these agents is compromised by the development of drug resistance in pathogen populations. Notably, the emergence of additional drug-resistant species, such as *Candida auris* and *Candida glabrata*, further threatens the limited armamentarium of antifungals available to treat these serious infections. Here, we describe our current arsenal of antifungals and elaborate on the resistance mechanisms *Candida* species possess that render them recalcitrant to therapeutic intervention. Finally, we highlight some of the most promising therapeutic strategies that may help combat antifungal resistance, including combination therapy, targeting fungal-virulence traits, and modulating host immunity. Overall, a thorough understanding of the mechanistic principles governing antifungal drug resistance is fundamental for the development of novel therapeutics to combat current and emerging fungal threats.

Graphical Abstract



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1. INTRODUCTION

Over the past several decades we have witnessed a concerning phenomenon where conventional antimicrobials are becoming increasingly ineffective at combatting infectious disease. With the current situation commonly being described as a postantibiotic era, there remains a great public health need for the development of novel therapeutics to combat pathogenic microbes, as well as a need to predict and prevent the evolution of resistance to our current armamentarium of antimicrobials. This issue of antimicrobial resistance is of particular concern in the context of fungal pathogens given the dearth of distinct classes of antifungals for treatment of invasive infections and the emergence and spread of multidrug-resistant fungal pathogens.

Despite the devastating impact that pathogenic fungi have on human health, they remain a relatively underappreciated contributor to human disease and mortality. Fungi infect billions and kill an estimated 1.5 million individuals annually, which is comparable to the mortality rates of more broadly recognized diseases such as tuberculosis or malaria.[1,2] The prevalence of serious diseases caused by fungi has surged in recent decades due to the increasing number of immunocompromised individuals, including cancer patients, organ transplant recipients, individuals infected with HIV, and the increasing elderly population.[3,4]

The predominant etiological agents of systemic fungal infections include species of *Candida*, *Aspergillus*, and *Cryptococcus*, which collectively account for over 90% of mycotic deaths.[1,3] *Candida* species are the most common cause of invasive mycotic disease in individuals who are severely immunocompromised, have endured invasive clinical procedures, or have experienced major trauma that requires extended treatment in intensive care units.[1,3,5] Specifically, *Candida* species, and in particular *Candida albicans*, rank as a leading cause of all healthcare-associated bloodstream infections in the United States, with a crude mortality rate of ~40% despite antifungal intervention.[1,5–7] In many healthy individuals, *C. albicans* is present as a harmless commensal in the oral cavity or the gastrointestinal tract; however, in severely immunocompromised patients, this fungus can disseminate into the bloodstream and colonize internal organs, resulting in life-threatening systemic infections.[1,8,9] Among non-*albicans* species, *Candida glabrata* is the most prevalent cause of invasive candidiasis, with a consistently increasing number of cases reported over the past several years.[5] Very recently, the emergence of *Candida auris* has caused great concern for medical practitioners and researchers across the globe. First isolated in 2009 from the external auditory canal of a patient in Japan,[10] *C. auris* has been reported in over 30 countries on 6 continents in less than a decade.[11] Phylogenetic analysis revealed four major clades of *C. auris* that cluster geographically and are distinguished by thousands of distinct single nucleotide polymorphisms (SNPs).[12] Distinguishing features of *C. auris* include its propensity to colonize the human skin, exceptional nosocomial persistence, and prevalence of resistance to at least one antifungal class.[13]

Despite the detrimental impact fungi have on human health, only a few classes of antifungal drugs are currently available for treating these life-threatening infections. In general, the development of novel antifungal agents has been slow largely due to the eukaryotic nature of fungal cells, challenges associated with compound permeability across the fungal cell

wall and membrane, and limited interest from the pharmaceutical industry in developing novel antifungals.[14–16] In fact, a new class of antifungals has not entered clinical practice since the mid-2000s.[15,17,18] Further threatening our limited repertoire of clinically relevant antifungals is the ever-increasing prevalence of fungal strains with intrinsic or acquired resistance to one or more drug classes. This Review will summarize our current arsenal of available antifungals, the resistance mechanisms that *Candida* species have employed to combat these agents, and potential new therapeutic approaches that can be explored to combat these pathogens.

2. CURRENT ARSENAL OF ANTIFUNGAL DRUGS

The polyenes are natural, amphipathic molecules that represent the oldest class of antifungal drugs used to treat systemic fungal infections. Introduced into medical practice in the 1950s, the most commonly used polyene, amphotericin B, exhibits potent activity against a broad spectrum of clinically relevant fungal species, including several species of *Candida*, *Aspergillus*, and *Cryptococcus*.[19,20] Traditionally, the polyenes were thought to directly bind to ergosterol, forming drug–lipid complexes that intercalate into fungal cell membranes, causing leakage of intracellular components and, ultimately, cell death.[20] However, recently this model was challenged by structural and biophysical studies, which revealed that amphotericin B forms extramembranous aggregates that extract ergosterol from fungal cell membranes, acting as a fungicidal “sterol sponge”[21] (Figure 1). Despite its broad-spectrum antifungal activity, the clinical use of amphotericin B is disfavored, primarily due to poor oral bioavailability and dose-dependent toxic effects toward the host, resulting from similarities between ergosterol and cholesterol.[20,22] In the mid-1990s, lipid formulations of amphotericin B were introduced that led to vast improvements in nephrotoxicity,[18,19] and as such these formulations have served as an alternative treatment option in cases where antifungal availability is restricted or resistance to other more suitable antifungals precludes their utility.[23] Fortunately, resistance to amphotericin B rarely occurs in *C. albicans* likely due to the severe fitness costs associated with the evolution of polyene resistance.[24,25] In clinical isolates of *C. auris*, resistance to amphotericin B can range from 0% to 30%, and preliminary evidence suggests that there is transient and inducible amphotericin B resistance in this species.[13]

First introduced in the 1980s, the azoles have served as the most frequently deployed drug class in the clinic. These heterocyclic synthetic compounds exert antifungal activity by blocking the synthesis of ergosterol, a key constituent of the fungal cell membrane, which leads to disruptions in membrane stability, permeability, and the function of membrane-bound enzymes.[18,19] Azole exposure also causes the accumulation of toxic sterol intermediates, including 14-α-methy-3,6-diol.[18,19] Specifically, the azoles inhibit the cytochrome P450 enzyme lanosterol 14-α-demethylase, encoded by *ERG11* in *Candida* species, by binding to the heme group in the active site[9] (Figure 1). In contrast to other antifungals, many azole drugs possess exceptional oral bioavailability and are available in both oral and intravenous formulations.[27] However, a significant drawback of azoles includes the high potential for interactions with other drugs as they also inhibit mammalian cytochrome P450 enzymes, which are responsible for drug metabolism.[14] To overcome this limitation, the development of new azoles (VT-1161, VT-1129, and VT-1598) that

possess higher specificity for fungal enzymes is currently underway.[28–31] An additional limitation is that azoles only inhibit the growth of *Candida* and *Cryptococcus* without killing these pathogens, imposing strong directional selection pressure and promoting the development of antifungal drug resistance. Susceptibility to azoles varies greatly among species of *Candida*. For the most commonly used azole fluconazole, *C. albicans* is generally susceptible, *Candida krusei* is intrinsically resistant, and resistance is steadily increasing for *C. glabrata*.[5,32] Alarmingly, for *C. auris*, fluconazole resistance is overwhelmingly abundant; for example, in a study of 350 clinical isolates collected from 10 hospitals in India, 90% of isolates exhibited reduced susceptibility to fluconazole.[33]

The only novel antifungal drug class to enter medical practice in decades is the echinocandins, which are natural product derivatives composed of a cyclic hexapeptide core with an N-linked fatty-acyl side chain. Echinocandins exert antifungal activity by disrupting the cell wall, a structure that is completely absent in mammalian cells. They bind non-competitively to the catalytic subunit of (1,3)-β-D-glucan synthase, which is encoded by *FKS1* in *C. albicans* and by both *FKS1* and *FKS2* in *C. glabrata* (Figure 1). A block in the biosynthesis of (1,3)-β-D-glucan culminates in a disruption of cell wall integrity and an imbalance in osmotic pressure, culminating in a fungicidal effect.[34] At present, echinocandins are recommended as a first-line treatment for invasive candidiasis given their potent activity against *Candida* species.[23,35] However, this drug class is clinically ineffective against species of *Cryptococcus*.[19] Major advantages of echinocandins include their excellent safety profile, greater fungal selectivity, and low potential for drug–drug interactions.[36] However, like polyenes, echinocandins exhibit poor oral absorption, and consequently, clinical use is limited to intravenous administration.[37] Recent work has advanced a structurally distinct 1,3-β-D-glucan synthase inhibitor, Ibrexafungerp, which is available in both oral and intravenous formulations and is currently in phase II and phase III clinical trials.[38] Given their widespread clinical use, resistance to echinocandins has inevitably emerged in recent years. Resistance has been most prominently recognized in *C. glabrata*; for example, in one U.S. hospital, the rate of echinocandin resistance in *C. glabrata* increased from 4.9% to 12.3% over a 10-year period.[39] Echinocandin resistance in clinical isolates of *C. auris* has also been documented.[13,33,40] Of utmost concern is the emergence of multidrug-resistant isolates of *C. auris*, which are impervious to all three antifungal drug classes, deeming these pathogens essentially untreatable.[13,33] Fortunately, for other clinically relevant *Candida* species, including *C. albicans*, susceptibility to the echinocandins remains high.[5]

3. MECHANISMS OF ANTIFUNGAL RESISTANCE

Our frequent and prophylactic use of antifungals has led to the development of robust resistance in many *Candida* species. The term resistance can be defined as a strain that has a minimal inhibitory concentration (MIC) for a particular antifungal above specific clinical breakpoints; resistance can also be used more broadly to indicate a strain with an increase in MIC to an antifungal drug relative to a control or reference strain.[41] This is in contrast to the term tolerance, which describes the ability of a drug-susceptible fungal strain to grow in the presence of an antifungal at concentrations above the MIC.[41] Numerous adaptive mechanisms of antifungal drug resistance and tolerance have been identified, including drug

target alteration or overexpression, upregulation of multidrug transporters, and activation of cellular stress responses (Figures 2 and 3). These mechanisms will be described in additional detail in the sections below.

3.1. Alterations in Drug Targets

3.1.1. Azoles.—In *C. albicans*, a common mechanism of azole resistance involves amino acid substitutions in the drug target, Erg11, which leads to lower drug-binding affinity for the lanosterol demethylase enzyme[15,42](Figure 2). Over 140 amino acid substitutions in Erg11 have been associated with azole resistance in this species,[43] with the majority of these substitutions clustered into hot-spot regions ranging from amino acids 105–165, 266–287, and 405–488.[44] Notably, only a few amino acid substitutions have been confirmed experimentally to confer azole resistance.[45,46] Molecular mapping of *C. albicans ERG11* variant positions revealed that mutations reside in the catalytic site of the enzyme, the fungus-specific external loop, and the proximal surface, as well as between the proximal surface and the heme.[47] Preliminary studies have also implicated *ERG11* mutations in azole resistance in *C. auris*. Whole-genome sequencing of 47 *C. auris* clinical isolates, of which the majority were resistant to fluconazole, identified three hot-spot amino acid substitutions previously implicated in azole resistance in *C. albicans*: Y132F, K143R, and F126L.[13,40] Consistent with these findings, another study of 44 *C. auris* clinical isolates detected amino acid substitutions at positions Y132 and K143 in *Erg11* in 77% of fluconazole-resistant strains.[33] Heterologous expression of the *C. auris ERG11* allele harboring either the Y132F or K143R substitution into the model yeast *Saccharomyces cerevisiae* resulted in elevated MIC values to both fluconazole and voriconazole, supporting the causal link between these mutations and *C. auris* azole resistance.[48] Interestingly, *ERG11* mutations appear to be strongly associated with specific geographic clades of *C. auris*, the K143R and Y132F substitutions are linked to isolates in the South Asian and South American clades, whereas the F126L substitution is associated with the African clade.[40] In contrast, for *C. glabrata*, alterations of the drug target have not been reported as a significant mechanism of azole resistance.[49–51] Indeed, only a single case of a fluconazole-resistant clinical isolate possessing an amino acid substitution in Erg11 has been documented to date.[52]

Overexpression of *ERG11* is common in azole-resistant clinical isolates of *C. albicans* and directly contributes to increased target abundance, ultimately lowering drug susceptibility[15,17](Figure 2). For *C. albicans*, the transcriptional activator, Upc2, is a crucial regulator of many ergosterol biosynthesis genes, including *ERG11*. Indeed, gain-of-function (GOF) mutations in *UPC2* cause the constitutive overexpression of ergosterol biosynthesis genes, a higher ergosterol content, and a reduction in fluconazole susceptibility.[53–58] Furthermore, disruption of *UPC2* in an azole-resistant clinical isolate abrogated *ERG11* overexpression and increased fluconazole susceptibility, highlighting the importance of this transcriptional activator in mediating *C. albicans* azole resistance.[59] Similarly, in *C. glabrata*, disruption of *UPC2A* in an azole-resistant clinical isolate reduced cellular ergosterol content and increased susceptibility to sterol biosynthesis inhibitors.[60]

3.1.2. Polyenes.—Resistance to polyenes is extremely uncommon; however, in the rare incidence that it does occur, it is mediated by alterations in enzymes that reduce

drug-binding affinity or deplete ergosterol from the membrane[61] (Figure 2). In *C. albicans*, reduced amphotericin B susceptibility can occur through mutations in several ergosterol biosynthesis enzymes, including *ERG2*,[62] *ERG3*,[63] *ERG5*,[64] and *ERG11*.[65] Likewise, for *C. glabrata*, mutations in *ERG2*,[52] *ERG6*,[66] and *ERG11*[52] have been documented in polyene-resistant clinical isolates. For *C. auris*, whole-genome sequencing of resistant clinical isolates following treatment with amphotericin B revealed higher expression of several genes in the ergosterol biosynthesis pathway (*ERG1*, *ERG2*, *ERG6*, and *ERG13*) compared to untreated controls and susceptible isolates.[12] Aside from genetic alterations, azole treatment has also been attributed to enhanced polyene resistance in *Candida* species, as a consequence of azole-mediated reduction in cellular ergosterol levels.[67]

3.1.3. Echinocandins.—For most *Candida* species, echinocandin resistance is primarily mediated by mutations in the *FKS* genes (Figure 2). In *C. albicans*, mutations that confer echinocandin resistance occur in the essential gene, *FKS1*. Hot-spot regions correspond to amino acids 641–649 (hot spot 1) and amino acids 1357–1364 (hot spot 2).[68] Mutations at these regions decrease the IC_{50} of the glucan synthase enzyme by several orders of magnitude, elevate MIC values, and result in cross-resistance to diverse echinocandins.[69–72] In *C. albicans*, serine 645 (S645) within hot-spot region 1 exhibits the highest frequency of substitution and is associated with the most prominent resistance phenotype.[70–72] For the related *FKS2* and *FKS3* genes, less is known about their contributions to echinocandin resistance in *C. albicans*; however, their deletion has been shown to result in higher *FKS1* transcript levels and lower echinocandin susceptibility.[73] In contrast to *C. albicans* and other yeasts, mutations conferring increased echinocandin resistance in *C. glabrata* occur in both *FKS1* and *FKS2* at regions homologous to those of *C. albicans*.[70,74] In fact, *C. glabrata* *FKS2* is expressed more highly than *FKS1*,[70] and mutations in *FKS2* outnumber *FKS1* mutations by two-to-one.[74] In echinocandin-resistant clinical isolates, the highest frequency of substitutions occurs at residue serine 663 in *FKS2*, which is equivalent to *C. albicans* S645.[70]

For *C. auris*, sequencing of *FKS1* in 38 *C. auris* clinical isolates revealed a mutation causing a phenylalanine substitution at serine 639 (S369F) (equivalent to S645 in *C. albicans*), which is associated with resistance to the echinocandins.[33] Furthermore, the clinical significance of this mutation was demonstrated using an in vivo murine model of invasive candidiasis. Mice challenged with a S639F mutant failed to respond to treatment with caspofungin in contrast to mice challenged with its susceptible wild-type counterpart.[75] Additionally, proline and tyrosine substitutions of the same S639 residue have been detected in *C. auris* echinocandin-resistant clinical isolates.[76]

3.2. Overexpression of Efflux Pumps

3.2.1. Azoles.—The upregulation of plasma membrane efflux pumps is a major mechanism governing azole resistance in many fungal pathogens. The first main class of efflux pumps implicated in azole drug resistance is the ATP-binding cassette (ABC) superfamily. ABC transporters possess two transmembrane-spanning domains and two cytoplasmic nucleotide-binding domains (NBDs). The NBD drives the movement of substrates across the fungal membrane via ATP hydrolysis.[77] The second class of efflux

pumps implicated in azole resistance is the major facilitator (MF) superfamily. Like the ABC superfamily, MF transporters also possess transmembrane-spanning helices but use the proton gradient generated across the plasma membrane to drive MF-mediated translocation.[77]

In *C. albicans*, overexpression of two homologous ABC transporters, Cdr1 and Cdr2, have been frequently implicated in azole resistance (Figure 2), particularly in patients receiving long-term antifungal therapy.[15,17] Of the two transporters, Cdr1 is the major determinant of azole resistance; deletion of *CDR1* in a clinical isolate reduces resistance to the azoles by 4-to 8-fold. However, deletion of *CDR2* typically results in a much weaker effect.[78] The transcription factor Tac1 (transcriptional activator of CDR) binds to cis-acting drug-response elements (DREs) in the promoters of *CDR1* and *CDR2* to regulate their expression. Indeed, single amino acid substitutions in the activation domain of Tac1 resulting in hyperactive alleles have been linked to azole resistance due to the constitutive overexpression of both *CDR1* and *CDR2*.[79] Constitutive upregulation of *CDR1* and *CDR2* also contributes to azole resistance in clinical isolates of *C. glabrata*. Genetic disruption of *CDR1* caused increases in azole susceptibility, and this effect was further exaggerated by the disruption of *CDR2*.[80,81] In addition to Cdr1 and Cdr2, a third ABC transporter, Snq2, plays an important role in *C. glabrata* azole resistance.[82] In *C. glabrata*, expression of these ABC transporters is mediated by the zinc cluster transcriptional activator, Pdr1 (pleiotropic drug resistance 1), which directly binds to the pleiotropic drug-response element (PDRE) in the promoter region of target genes.[83,84] Indeed, the most prevalent mechanism of clinical azole resistance in *C. glabrata* involves *PDR1* gain-of-function mutations that result in the upregulation of its downstream target transporters.[85–87] Specifically, engagement with the KIX domain of the Mediator coactivator subunit Gal11A (also known as Med15A) is essential for the upregulation of *C. glabrata* Pdr1-target genes.[88] More recently, in *C. albicans*, the Mediator complex was shown to play an important role in Tac1-mediated azole resistance, as deletion of the Mediator tail module in Tac1 gain-of-function mutants reduced *CDR1* transcription and increased fluconazole susceptibility.[89]

Although 95 MF transporters are encoded in the *C. albicans* genome,[90] fluconazole resistance has only been linked to Mdr1 (multidrug resistance 1)[91,92] (Figure 2). Expression of this MF pump is regulated by the transcription factor, Mrr1 (multidrug resistance regulator 1), such that deletion of *MRR1* abolishes *MDR1* expression and increases susceptibility to fluconazole,[93] whereas activating point mutations in *MRR1* increase azole resistance.[94] Interestingly, deleting *MRR1* reduces fluconazole resistance to a greater extent than the deletion of the *MDR1* efflux pump itself, suggesting that *MRR1* may regulate additional determinants of azole resistance.[93] More recently, the *C. albicans* Swi/Snf chromatin remodelling complex was implicated as a major coactivator of *Mrr1* as deletion of the catalytic subunit of the Swi/Snf complex, *SNF2*, in *MRR1* gain-of-function strains led to drastic reductions in both Mdr1 activation and fluconazole resistance.[95] In contrast to *C. albicans*, the *C. glabrata* homologue of *MDR1, FLR1*, is not significantly associated with azole resistance,[96] as is the case with several other non-*albicans Candida* species.[77]

In *C. auris*, the genome encodes for many transporters orthologous to those in *C. albicans* that primarily belong to the ABC and the MF families.[12,97] Phylogenetic analysis revealed

that *C. auris* possesses homologues of *MDR1*, *CDR1/CDR2*, and *CDR4*, as well as two copies of *SNQ2*.[12] Several lines of evidence have indicated the important relationship between efflux pumps and azole resistance in *C. auris*. Specifically, one study observed that, although *CDR1* and *MDR1* were both overexpressed in azole-resistant *C. auris* isolates, only the deletion of *CDR1* led to significant reductions in azole MIC values.[98] The importance of this efflux pump was confirmed in a separate study in which the deletion of *CDR1* was sufficient to confer an 8-fold increase in fluconazole sensitivity.[99] Efflux-mediated mechanisms of azole resistance also appear to play an important role in *C. auris* biofilms. Transcriptomic analysis of drug-resistant *C. auris* biofilms revealed the upregulation of both ABC and MF transporters, and treatment with efflux pump inhibitors increased fluconazole sensitivity by 4- to 16-fold, highlighting the significance of efflux activity in biofilm-mediated resistance.[100] In addition to drug transporters, numerous transcription factors are also present in the *C. auris* genome, including two copies of both *TAC1* and *MRR1*,[12] suggesting that differences in transcriptional regulatory machinery may serve as a mechanism by which efflux pumps are transcribed at a higher constitutive level in *C. auris*. Recently, a study demonstrated that mutations in *TAC1B* arise rapidly in vitro upon exposure to fluconazole and that resistance-associated *TAC1B* mutations are present among several fluconazole-resistant *C. auris* clinical isolates.[101] Interestingly, one of the two copies of *MRR1* is truncated at its carboxy terminus, a common regulatory domain, raising the question as to whether *C. auris MRR1* has altered regulation relative to homologues in other *Candida* species.[102]

3.2.2. Polyenes and Echinocandins.—

In contrast to azoles, the upregulation of efflux pumps plays a rather insignificant role in conferring resistance to the echinocandins. This is consistent with the fact that echinocandins function at the outer leaflet of the fungal cell membrane,[18,19] as well as the fact that these large cyclic hexapeptide molecules are likely to be poor substrates of fungal efflux pumps due to their size and hydrophobicity.[103] Similarly, for polyenes, the lack of efflux-mediated mechanisms of resistance may be attributed to their inability to enter the drug-binding pocket of transporters.[103] Interestingly, in *C. auris*, a recent study highlighted the potential involvement of drug transporters in amphotericin B resistance. Whole-genome sequencing of polyene-resistant clinical isolates led to the identification of four non-synonymous mutations, one of which was in a putative membrane transporter. Notably, none of the SNPs were found in the coding sequences of ergosterol biosynthesis genes, which is the conventional mechanism of polyene resistance in other species of *Candida*.[104] Although *C. auris* appears to possess a rather unusual mechanism of amphotericin B resistance, the manner by which membrane transporters govern this process requires further analysis.

3.3. Modulation of Stress Responses

The diverse and dynamic niches that fungal pathogens inhabit are subject to a variety of environmental fluctuations, including temperature, pH, and nutrient levels, which are capable of perturbing cellular homeostasis and imposing significant stress on the fungal cell. Antifungal agents represent a chemical stressor these pathogens must recognize, respond to, and adapt to in order to survive.[17,105] Consequently, fungal pathogens have evolved broad

stress-response circuitry that enable them to thrive in the presence of diverse cellular insults (Figure 3).

3.3.1. Azoles.—A key mechanism through which *C. albicans* develops resistance to the azoles that is contingent upon stress responses is through alteration of the ergosterol biosynthesis pathway. Loss-of-function mutations in *ERG3*, which encodes a Δ−5,6-desaturase, block the cellular accumulation of 14-α-methyl-3,6-diol, the toxic sterol intermediate that is otherwise produced as a result of Erg11 inhibition by the azoles.[106] Alternatively, 14-α-methyl fecosterol is incorporated into the fungal cell membrane, allowing for continued growth and replication in the presence of azoles. Azole resistance in *C. albicans* has been associated with five missense mutations in *ERG3* (A168V, S191P, G261E, T329S, and A353T) and two further nonsense mutations (Y325* and Y190*), leading to loss of function.[51,107]

A global cellular regulator governing stress responses in diverse fungal pathogens is the essential molecular chaperone, heat shock protein 90 (Hsp90).[108,109] Hsp90 is highly abundant, and its function is tightly coupled to environmental perturbations. It interacts with over 20 cochaperones that facilitate the recognition of specific client proteins, which are enriched in kinases, signal transducers, and transcription factors, many of which serve as hubs in regulatory networks.[110–112] These unique attributes enable Hsp90 to serve as both a capacitor for the storage and release of genetic variation and a potentiator to allow for the rapid emergence of new traits.[111,112] Specifically, Hsp90 potentiates the rapid evolution of azole resistance in *C. albicans*, as well as in more evolutionarily distinct fungi, as genetic or pharmacological inhibition of Hsp90 reduces azole tolerance and abrogates resistance.[113,114] Primary investigations of Hsp90-mediated resistance in *S. cerevisiae* revealed that azole resistance acquired via loss-of-function of Erg3 is Hsp90-dependent.[114] In *C. auris*, Hsp90 also governs azole tolerance in azole-susceptible strains, highlighting its role in orchestrating cellular responses upon exposure to membrane stress in this pathogen.[99] Notably, Hsp90 does not mediate azole resistance in *C. albicans* or *C. auris* isolates where azole resistance is caused by the overexpression of efflux pumps, highlighting this as an Hsp90-independent mode of resistance.[99,114]

The mechanisms through which Hsp90 confers antifungal resistance are complex given its global impact on cellular signaling. A key Hsp90 client that mediates its effects on antifungal drug tolerance and resistance is the calcium-calmodulin activated protein phosphatase calcineurin.[114,115] Azole treatment activates calcineurin-dependent stress responses in *C. albicans*,[115] and genetic or pharmacological impairment of the phosphatase renders *C. albicans* hypersensitive to the azoles.[116] Hsp90 inhibition blocks azole activation of the calcineurin-dependent stress response and phenocopies the effects of calcineurin inhibition,[115] highlighting the interconnectedness between calcineurin and Hsp90 in regulating azole tolerance and resistance. The *C. albicans* protein kinase C, Pkc1, also regulates cellular responses to the azoles through a MAPK cascade consisting of Bck1, Mkk1/2, and Mkc1.[117] Compromise of PKC-MAPK signaling phenocopies both Hsp90 and calcineurin inhibition, reducing azole-resistance phenotypes in distinct *C. albicans* clinical isolates.[117] Moreover, genetic depletion of Hsp90 in *C. albicans* destabilizes Mkc1, Bck1,

and Pkc1, ultimately blocking PKC signaling.[117] Thus, Hsp90 regulates basal tolerance and resistance to membrane-perturbing azole antifungals through multiple signaling cascades.

In addition to Pkc1, a number of other kinases are involved in stress-response circuity that enables the survival of *C. albicans* during azole-induced stress. Interestingly, many of these signal transducers also have connections with Hsp90 and other regulators discussed earlier. One promiscuous kinase involved in azole resistance is casein kinase 2 (CK2). Overexpression of the catalytic subunit of the CK2 complex, *CKA2*, confers fluconazole resistance that is dependent on calcineurin signaling.[118] Further, chemical-genetic screens in *C. albicans* revealed that CK2 subunits genetically interact with Hsp90, and additional biochemical characterization established that CK2 phosphorylates Hsp90 to regulate its chaperoning activity.[119,120] Other kinases important for cellular responses to azoles include target of rapamycin (TOR) kinases, which are involved in nutrient sensing and signaling, as well as coupling cellular growth and metabolism to environmental cues. Compromising TOR function with the pharmacological inhibitor rapamycin abrogates *erg3*-mediated resistance to the azoles.[121] Further, the cyclic hexadepsipeptide natural product beauvericin potentiates azoles in *C. albicans*, *C. neoformans*, and *A. fumigatus*.[122] Mechanistic studies highlighted a dual mechanism of action of beauvericin in which it both blocks multidrug efflux and inhibits the Tor1 kinase, thereby activating CK2 and inhibiting Hsp90 function.[122] Hyper-activation of TOR signaling in fungal pathogens has also been shown to bypass azole toxicity via Hsp90-dependent calcineurin stabilization.[123] Thus, fungal kinases play complex and pivotal roles in governing responses to azole-induced cell membrane stress.

Post-translational modifications including methylation, acetylation, and phosphorylation also regulate stress responses, virulence, and antifungal tolerance in *Candida* species.[15,17,19] Specifically, reversible acetylation by various lysine acetyl-transferases (KATs) and lysine deacetylases (KDACs) is important in facilitating chromatin-mediated transcriptional regulation, as well as the post-translational regulation of many cellular proteins. Inhibition of many of these enzymes decreases basal tolerance and resistance to antifungal drugs. For instance, the KDACs Hda1 and Rpd3 deacetylate Hsp90 in *S. cerevisiae*, and genetic deletion of these factors increases azole sensitivity and impairs the evolution of azole resistance.[124] In *C. albicans*, there is extensive functional redundancy as KDACs Hos2, Hda1, Rpd3, and Rpd31 all contribute to azole resistance.[125] Further, a molecule thought to inhibit the KDAC Hos2, MGCD290 (Mirati Therapeutics, San Diego, CA, U.S.A.), displays synergistic activity with azoles against diverse *C. albicans* drug-resistant clinical isolates.[126,127] Furthermore, in vivo studies coupled with preliminary clinical trials have supported the use of MGCD290 in combination with fluconazole to treat *C. albicans* infections.[128] Chromatin remodeling and the activity of the sirtuin-family of KDACs have also been shown to mediate tolerance to the azoles in *C. glabrata*, where inactivation results in enhanced susceptibility to azoles and other cellular stressors.[129] KATs also modulate azole resistance. Genetic impairment of *C. albicans ADA2*, which encodes a component of the Spt-Ada-Gcn5-acetyl-transferase (SAGA) coactivator complex, confers hyper-sensitivity to fluconazole due to impaired upregulation of efflux pumps Cdr1 and Mdr1.[130]

Environmental pH plays a role in antifungal tolerance as growth of *C. albicans* at an acidic pH reduces growth observed at azole concentrations above the MIC.[131] In yeast, pH signaling is mediated by the highly conserved Rim pathway, where external pH is detected by the transmembrane proteins Rim21 and Dfg16, which signal through a protein cascade consisting of Rim8, Rim13, Rim20, and the terminal transcription factor Rim101.[9] Rim101 regulates the expression of genes involved in cell wall, yeast-to-hyphal transition, adhesion, iron metabolism, and biofilm formation. Phenotypic and transcriptional analyses have implicated multiple components of the Rim pathway in azole tolerance and identified that Hsp90 is a downstream effector of Rim signaling, likely contributing to Rim-mediated effects on antifungal tolerance.[132]

3.3.2. Echinocandins.—Similar to the azoles, exposure to the cell wall targeting echinocandins initiates a number of interconnected and adaptive responses that modulate fungal susceptibility to this antifungal class. Echinocandin-induced defects in the cell wall are detected by transmembrane proteins that facilitate the activation of the GTPase Rho1. Downstream Rho1 targets include Pkc1 and the β−1,3-glucan synthase subunits.[133–135] Cell wall salvage mechanisms can then be activated through these and other signaling networks. The fungal cell wall has dynamic and compensatory capabilities to increase production of one or more components upon inhibition of another. In vitro assessment of echinocandin-mediated β−1,3-glucan inhibition revealed that *C. albicans* facilitates the compensatory upregulation of chitin synthesis and redistribution of polysaccharides in the cell wall during times of cell wall stress.[136,137] This response contributes to the paradoxical growth phenomenon observed at high echinocandin concentrations.[138,139] Three signal transduction pathways, the PKC-MAPK pathway, the Ca^{2+}-calcineurin pathway, and the high-osmolarity glycerol (HOG) pathway coordinately regulate chitin synthesis to maintain cell wall integrity.[140] PKC-MAPK and calcineurin signaling are also critical in establishing basal echinocandin tolerance through elevated chitin production in *C. glabrata*.[141,142]

As discussed, Hsp90 regulates the function of calcineurin, as well as a number of stress-activated protein kinases, which is crucial in mediating responses to the echinocandins.[113,143,144] Pharmacological or genetic impairment of Hsp90 function potentiates echinocandin activity in *C. albicans*, *C. glabrata*, and the distantly related pathogenic mold *A. fumigatus*.[115,142,145] Furthermore, inhibition of Hsp90 reduces echinocandin resistance in *C. glabrata* clinical isolates with mutations in the echinocandin target gene *FKS1*.[142] Proof-of-principle experiments established that genetic depletion of *C. albicans* Hsp90 in vivo enhances the efficacy of the echinocandins in a systemic murine model of infection, and pharmacological inhibition of Hsp90 enhances echinocandin efficacy in a *Galleria mellonella* model of *A. fumigatus* infection.[113,115] These effects are in part attributed to the role of Hsp90 in stabilizing calcineurin and components of the PKC-MAPK pathway, as deletion of these signal transducers results in decreased chitin synthesis upon echinocandin exposure and increased echinocandin susceptibility.[140]

An expanded repertoire of cellular factors influencing fungal tolerance to the echinocandins include the *C. albicans* transcription factor Cas5. Initially identified in a genetic screen for mutants hypersensitive to caspofungin,[146] homozygous deletion of *CAS5* was shown to reduce Fks1-mediated echinocandin resistance.[147] Under basal conditions, Cas5 regulates

a distinct set of transcriptional targets under basal physiological conditions compared with those in response to cell wall stress, with a crucial role in transcriptional regulation of cell wall homeostasis and cell cycle dynamics.[147] This unveiled novel regulatory circuitry through which stress responses, cell cycle regulation, and antifungal resistance were coupled.[147] Further functional genomic screening efforts implicated proteins of the eukaryotic chaperonin containing TCP-1 (CCT) complex in modulating cellular responses to echinocandin-induced stress, enabling both tolerance and resistance.[144] The CCT complex is involved in the assembly and folding of cytoskeletal proteins, and its depletion results in actin perturbation and cell wall stress. Hsp90 associates with the CCT complex,[144] indicating that it is a member of the Hsp90 interaction network with potential to be targeted to combat echinocandin resistance.

3.3.3. Polyenes.—Resistance to the polyenes in *Candida* species is a rare phenomenon given the fitness costs associated with the acquisition of resistance. Clinical isolates of *C. albicans* with loss-of-function mutations in *ERG3* have demonstrated cross-resistance to the polyenes and azoles due to their marked reductions in ergosterol content.[25,106] The fitness and survival of amphotericin B-resistant *Candida* isolates are critically dependent upon Hsp90 expression and function. As a consequence, pharmacological inhibition of Hsp90 in resistant *C. albicans* or *C. tropicalis* strains abolished amphotericin B resistance.[25] These resistant mutants also exhibited constitutive activation of diverse stress responses, with a genetic dependency of resistance on stress-response regulators, including calcineurin, Pkc1, and Hog1.[25] It has been postulated that resistance to amphotericin B may enable pathogens to efficiently cope with oxidative stress. *C. albicans* mounts a compensatory antioxidant response upon exposure to amphotericin B, and studies with amphotericin B-resistant *Candida* demonstrate significant reductions in reactive oxygen species (ROS) accumulation, reductions in protein carbonylation, reductions in basal mitochondrial respiration, and significant increases in catalase production.[148,149]

3.4. Genomic Modifications

Fungal pathogens possess remarkable genomic plasticity, allowing them to adapt to environmental perturbations and acquire antifungal resistance. Large-scale genomic alterations including aneuploidies, loss of heterozygosity (LOH), and chromosomal rearrangements can impact the expression of drug targets, efflux pumps, and other factors that contribute to resistance. The emergence of aneuploidy provides a unique opportunity for cells to rapidly develop genotypic and phenotypic diversity without permanently committing to the mutant genotype.[150,151] The fungistatic azoles cause alterations in DNA content and cell morphology through an ordered series of aberrant cell cycle events that select for aneuploidy formation.[152] Karyotype variability conferring azole resistance has been extensively studied in *C. albicans* and is commonly observed in both resistant clinical isolates and laboratory strains. A specific aneuploidy on the left arm of chromosome 5 is the most prevalent.[153,154] This common segmental aneuploidy consists of an isochromosome composed of two identical left arms of Chr5 flanking a centromere (i(5L)). Gain and loss of i(5L) tightly correlates with the gain and loss of azole resistance. The resistance associated with i(5L)-carrying isolates is mediated by increased copy numbers of *ERG11* and *TAC1*, as well as gain-of-function mutations and LOH of these resistance determinants.[155] Variant

forms of i(5L) also exist, including those that incorporate a region of chromosome 3 containing *MRR1*, allowing for further resistance to develop.[156]

Additionally, aneuploidies of chromosomes 3, 4, and 6 have been implicated in azole resistance in *C. albicans*.[157–159] Experimental evolution in the presence of azoles and the calcineurin inhibitor FK506 revealed extensive aneuploidies in strains that evolved resistance to the drug combination.[158] The aneuploidy common in all resistant lineages was an increased copy number of chromosome 4, suggesting that this chromosome may harbor important resistance determinants.[158] Further, experimental evolution in a strain sensitized to azoles due to loss of the GTPase activator protein Rgd1 resulted in an amplification of both chromosome 7 and a large segment of chromosome 3, which accompanied suppression of the azole-sensitivity phenotype; overexpression of a transporter gene on the affected region of chromosome 3 was sufficient to confer the suppression phenotype.[160] Although most extensively studied in *C. albicans*, the association between aneuploidy formation and fluconazole resistance in *Candida* species has also been described in *C. glabrata*. Similar to *C. albicans*, an increase in *ERG11* copy number has been observed in *C. glabrata* through the formation of aneuploidies and novel chromosomal configurations.[161,162]

LOH can also have a profound impact on drug resistance and is common in genomic regions containing determinants of azole susceptibility. LOH events can occur over short regions or over entire chromosomes. In *C. albicans*, LOH for the transcription factors *TAC1* and *MRR1* have been reported in azole-resistant isolates.[79,94] For both *TAC1* and *MRR1*, LOH can generate two copies of a hyperactive allele, which increases the expression of genes encoding Cdr1 and Cdr2, or Mdr1, respectively.[79] Constitutive upregulation of *ERG11* has also been reported as a consequence of LOH in the transcription factor *UPC2* in *C. albicans*.[54] Interestingly, work has shown that a plethora of stress conditions increase mitotic recombination rates in *C. albicans*, leading to an increase in LOH rates.[163] This suggests a mechanism by which *C. albicans* can achieve greater genotypic diversity upon exposure to environmental perturbations, which can have important consequences given the lack of a conventional sexual cycle in this organism.

The incidence of echinocandin and polyene resistance arising from chromosomal abnormalities is infrequent compared to azole resistance in *C. albicans*. Nevertheless, rare genomic alterations have been reported in echinocandin-resistant isolates. Stepwise acquisition of micafungin resistance by homozygous hot-spot mutations in *FKS1*, followed by LOH, was identified as a mechanism of echinocandin resistance in *C. albicans*.[164] Additionally, while an increase in chromosome 5 copy number confers azole resistance, a chromosome 5 monosomy causes decreases in β−1,3-glucan, increases in chitin, and decreases in echinocandin susceptibility.[165] Finally, trisomy of chromosome 2 reduces the susceptibility of *C. albicans* to caspofungin.[166] Adaptation to caspofungin in these strains is mediated by a calcineurin-mediated mechanism that is independent of the downstream transcriptional regulator Crz1.[166]

Substantial karyotype variability has been observed in *C. auris* under stress conditions, and preliminary genomic investigations revealed that isolates harbor between 5 and 7 chromosomes of varying sizes.[167] Although the relationship between karyotype, drug

resistance, and clade affiliation has not been determined, future investigations as to the impact of genomic plasticity on drug resistance for this emerging pathogen will no doubt lead to fascinating insights. *C. auris* undergoes replicative aging caused by asymmetric cell division. Interestingly, older cells exhibit higher tolerance to fluconazole, micafungin, and amphotericin B compared to younger cells.[168] Transient gene duplication in old *C. auris* cells increases expression of drug-resistance determinants, including *ERG11* and *CDR1*.[168] Whether the gene duplications observed resulted from chromosomal duplications remains inconclusive.[168] Overall, further mapping of *C. auris* chromosomes to investigate resistance patterns is required in order to more effectively understand the impact of genomic rearrangements on antifungal resistance in this emerging pathogen.

3.5. Intrinsic Antifungal Resistance

Thus far, we have focused on mechanisms through which fungal pathogens acquire antifungal drug resistance. However, a number of fungi display primary or intrinsic resistance to clinically available antifungals. 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.[169] Uncovering mechanisms governing intrinsic resistance is an important step to better understand and combat the clinical threat posed by these pathogens.

Intrinsic resistance to the azoles has been reported in several non-*albicans Candida* species, including *C. glabrata*, *C. krusei*, and *C. auris*. *C. glabrata* isolates demonstrate striking intrinsic resistance to fluconazole.[170] Even without prior azole exposure, *C. glabrata* isolates develop robust, stable resistance phenotypes within 4 days of in vitro exposure.[171] This is largely attributed to enhanced drug efflux through the overexpression of ABC transporters Cdr1, Pdh1, Yor1, and Snq2, which are all regulated by the transcription factor Pdr1.[80,82,170] Furthermore, *C. glabrata* "petite mutants", or strains with a mitochondrial DNA deficiency, exhibit upregulation of ABC transporters and higher levels of azole resistance.[86,172] Finally, *C. glabrata* can grow with altered membrane sterol composition and can facilitate sterol uptake in both aerobic and anaerobic conditions, as well as in the presence of fluconazole.[173] Active transport of exogenous sterols has been attributed to *C. glabrata*'s capacity to overcome fluconazole-mediated blocks in ergosterol biosynthesis.[173] For *C. krusei*, the mechanisms responsible for innate azole resistance are poorly understood. A number of likely contributors have been suggested, including the variable susceptibility of *C. krusei* Erg11 to inhibition by azoles, as well as increased efflux pump expression.[174,175] For example, *C. krusei* constitutively expresses the ABC transporter Abc1, which, when pharmacologically inhibited, results in sensitization of the pathogen to the azoles.[174] Finally, the remarkable azole resistance exhibited by *C. auris*, with over 90% of identified isolates displaying MICs above clinical breakpoints, garnered initial suspicions of robust intrinsic azole resistance in this species.[11,13,102] Amino acid substitutions in the azole target Erg11 that mediate azole resistance in *C. albicans*, including Y132F and K143R, were found in resistant *C. auris* isolates.[33] Further endeavors are still crucial to determine the reservoir and evolutionary trajectory of *C. auris* in order to uncover the mechanism driving the development of such widespread azole resistance in this pathogen.[11,13,102]

In addition to *Candida* species displaying intrinsic resistance to azoles, select strains and species also show inherent resistance to the echinocandins. *Candida parapsilosis* is an example of a species with intrinsically reduced susceptibility to the echinocandins.[176] Breakthrough *C. parapsilosis* infection has been reported during caspofungin therapy in humans, highlighting the clinical significance of this resistance phenotype.[177] *C. parapsilosis*, along with its sibling species *Candida orthopsilosis* and *Candida metapsilosis*, possesses a naturally occurring proline-to-alanine amino acid substitution in Fks1, which results in elevated resistance to echinocandins.[72] This substitution at position 660 is conserved and distal to hot-spot region 1 of the protein, causing a decreased affinity of the *C. parapsilosis* glucan synthase to caspofungin.[72] Mutations in *C. albicans* and *C. glabrata* at the equivalent position cause comparable increases in echinocandin resistance and decreases in enzyme affinity to the echinocandins.[72] Interestingly, *C. parapsilosis* isolates harboring the P660A substitution in Fks1 also demonstrate higher cell wall chitin levels compared to isolates with alternative Fks1 sequences, suggesting that alterations in cell wall composition accompanying this naturally occurring mutation may be important in intrinsic echinocandin resistance.

Antifungal resistance can be intrinsic to fungal species, as well as to distinct growth states adopted by typically nonresistant species. In most natural environments, *Candida* grows as biofilms, which are organized, three-dimensional microbial communities irreversibly attached to surfaces.[178] *Candida* biofilms are composed of a layer of yeast cells facilitating surface adherence, a heterogeneous layer of filamentous cells, and an outer layer composed of an exopolymeric matrix.[178,179] *C. albicans* is the fungal pathogen most commonly associated with biofilm formation, particularly as medical implants support its colonization. Although planktonic *C. albicans* cells are not intrinsically resistant to clinically available antifungals, *C. albicans* biofilms exhibit inherent multidrug resistance.[178,180,181] Cells comprising these biofilms overexpress efflux-pump encoding genes and are capable of expressing *CDR1* and *MDR1* during all developmental phases, which contributes to their robust resistance to azoles.[181,182] Glucan synthesis by Fks1 is critical for biofilm-mediated drug resistance, as β–1,3 glucan in the adhesive biofilm matrix sequesters antifungals in a “sponge” to prevent the drugs from reaching their targets.[183,184] This mechanism has been implicated in resistance to azoles, echinocandins, and polyenes.[15,184,185] Interestingly, Hsp90 and downstream factors of the PKC-MAPK pathway regulate β–1,3 glucan biosynthesis in the biofilm matrix,[184–186] and genetic or pharmacological compromise of Hsp90 restores azole efficacy in a rat catheter model of biofilm infection.[186] With the ever-growing increase in immunocompromised patients utilizing medical implants, it is particularly critical to develop novel strategies to efficiently combat biofilm-mediated infections.

4. THERAPEUTIC STRATEGIES TO COMBAT RESISTANCE

Although advances are being made to improve our current antifungal arsenal and to identify novel drug targets, the rate at which antimicrobials are being developed is vastly outpaced by the rate at which resistance is emerging. Thus, examining alternative approaches, beyond targeting essential genes or cellular functions, for the development of new antifungal treatments is imperative to bolster the antifungal pipeline. A variety of strategies are in the

early stages of being explored, including the use of combination therapy, the development of antivirulence agents, and modulating host immune responses to fungal pathogens to combat or prevent infection.

4.1. Combination Therapy

Combination therapy is routinely implemented for the treatment of diverse infectious diseases, including malaria, tuberculosis, and HIV/AIDS, while its potential in antifungal treatment is only beginning to be explored.[19,187] Specific drug combinations can overcome existing resistance and/or delay or prevent further resistance from emerging through numerous modalities (Figure 4). For instance, by combining multiple drugs with distinct mechanisms of action, the number of intracellular processes targeted increases, which makes evolution of resistance more difficult as the pathogen must acquire mutations in multiple genes.[17,187] Additionally, combination therapy can increase the fungicidal efficiency of normally fungistatic drugs (e.g. the azoles), reducing pathogen population sizes and, thus, the probability of resistance arising.[187,188] Importantly, in rare cases a phenomenon known as selection inversion can occur, reversing drug resistance by neutralizing selective advantages and imposing direct fitness costs on the evolution of drug resistance.[189] Finally, combination therapy has the potential to unveil additional efficacious treatments given that fungal biology is controlled by highly interconnected and functionally redundant networks of biological interactions.[190–192] The potential for targeting synthetic lethal interactions provides an expanded target space for efficacious combinations of compounds that on their own have little impact on fitness but combined are lethal. Together, this highlights that implementation of combination therapy is a beneficial avenue to address and treat drug-resistant fungal infections.

Discovery of molecules that target fungal resistance determinants to be used in combination with existing antifungals is an important strategy for the development of antifungal combination therapy. As discussed previously, upregulation of drug efflux is a conserved evolutionary mechanism that fungal pathogens employ to acquire resistance to the azoles, with the consequence that inhibition of efflux should improve azole susceptibility in such isolates. Studies in *C. glabrata* identified a small molecule termed iKIX1 that is able to bind the *C. glabrata* Gal11 KIX domain and prevent Pdr1-mediated gene activation, sensitizing previously resistant isolates to the azoles[193] (Figure 4). In vivo analysis using both *G. mellonella* and mouse models of *C. glabrata* infection demonstrated improved therapeutic efficacy of fluconazole when combined with iKIX1 during treatment.[193] Such analyses emphasize the therapeutic potential of small-molecule efflux inhibitors for use in the treatment of azole-resistant fungal infections.

In addition, inhibition of stress-response regulators confers therapeutic benefits in combination with existing antifungal drugs and can overcome preexisting resistance. Although Hsp90 is a global regulator of stress-response circuitry, making it an attractive target in antifungal combination therapy, it is also remarkably conserved across all eukaryotes. Therefore, use of generalist Hsp90 inhibitors, many of which have been explored for use as anticancer agents, causes prohibitive host side effects in the context of systemic fungal infection.[113] However, recent work focused on the *C. albicans* Hsp90

nucleotide-binding domain revealed that, despite Hsp90 sequence conservation across species, *C. albicans* Hsp90 possesses distinct conformational flexibility.[194] This observation enabled the synthesis of the first fungal-selective Hsp90 inhibitor, a resorcylate natural products derivative, as a proof-of-concept that efficiently inhibited *C. albicans* growth, potentiated azole activity against resistant isolates, and demonstrated low mammalian cell toxicity[194] (Figure 4). Building on this effort to produce fungal-selective Hsp90 inhibitors, a series of aminopyrazole-substituted resorcylate amides were synthesized and found to possess potent and fungal-selective Hsp90 inhibitory activity.[195] Similar approaches to selectively design inhibitors of fungal stress responses have been applied to the protein phosphatase calcineurin. Crystal structures of calcineurin complexed with the well-characterized inhibitor FK506 and the FK506-binding protein (FKBP12) from human and fungal pathogens revealed conformational differences.[196] This, along with additional biochemical characterization, led to the characterization of a less immunosuppressive FK506 analogue, APX879, with an acetohydrazine substitution of the C22-carbonyl of FK506. Intriguingly, APX879 exhibited reduced immunosuppressive activity while maintaining broad-spectrum antifungal activity and efficacy in a murine model of invasive fungal infection.[196] Such observations highlight the promise of leveraging chemo-structural approaches to synthesize fungal-selective molecules targeting highly conserved proteins.

In addition to the design of fungal-selective molecules that target known mediators of antifungal tolerance and resistance, a complementary and unbiased approach to identify synergistic drug interactions utilizes high-throughput screening of large compound collections. Molecules that enhance the activity of established antifungals against *S. cerevisiae*, *C. albicans*, and *C. neoformans* have been identified by chemical biology screens[197,198] and computational methods.[199,200] Many of these studies have not surprisingly observed that compounds that increase azole efficacy often target membrane homeostasis or sphingolipid biosynthesis.[201] However, select studies have also used chemical screens to uncover novel cellular targets with previously unappreciated roles in antifungal resistance. For example, recent work described the characterization of a 2,3-aryl-pyrazolopyridine scaffold compound, GW, as an inhibitor of the casein kinase 1 family member Yck2.[202] Combination of GW with caspofungin eradicated *C. albicans* drug resistance in vitro, and genetic depletion of *YCK2* caused a significant decline in fungal burden in a mouse model of systemic caspofungin-resistant *C. albicans* infection. This establishes Yck2 as an important mediator of cell wall integrity and echinocandin resistance[202] (Figure 4).

4.2. Targeting Virulence

Targeting fungal virulence provides a complementary approach to the development of fungicidal and fungistatic agents, as in principle these therapeutics simply aim to occlude the ability of a microbe to cause harm to its host. Although the potential utility of targeting nonessential genes has only recently been appreciated, there are already examples of antibiotics that target essential structures rather than essential proteins, as with daptomycin and the Gram-positive cell wall.[203] Targeting virulence factors offers many benefits including expanding the repertoire of antifungal targets, minimizing effects on the host mycobiome, and reducing selection pressure for the evolution of drug resistance.[204,205]

Among the *C. albicans* virulence factors, its capacity to undergo a morphogenetic transition between yeast and filamentous states is one of the most critical[206,207] (Figure 5). While the yeast morphology is important in facilitating early adhesion and dissemination events during *C. albicans* infection, the filamentous state is responsible for causing physical damage to tissues, systemic invasion, and immune evasion. Filamentation is regulated by numerous, complex signaling pathways, and the identification and analysis of small molecules that target essential components of this developmental transition have been promising in uncovering novel antivirulence agents. For instance, the small-molecule filastatin inhibits filamentous growth, biofilm formation, and pathogenesis in vivo[208] (Figure 5). Initial investigations of the clinical utility of this small molecule have suggested that using it to coat medical devices can prevent fungal adherence and disrupt biofilm formation, presenting a new clinical option to prevent acute nosocomial infections resulting from medical implant use.[209]

Intriguingly, many compounds that display bioactivity alone or in combination with conventional antifungals also modulate *C. albicans* morphogenesis. In fact, even the azoles directly impact *C. albicans* morphology by preventing the formation of hyphae.[9,210] In addition, targeting Hsp90 locks cells in a filamentous state, impairs the dispersal of *C. albicans* biofilms, and attenuates virulence in mouse models of infection.[186,211] Hsp90 has also been shown to regulate virulence programs in *Cryptococcus* and *Aspergillus* species.[145,212] In addition, the natural product beauvericin, which targets drug efflux and TOR signaling, also represses the expression of many filament-specific genes, blocking the yeast-to-hyphal transition.[122] Finally, Pkc1 not only plays pivotal roles in both echinocandin and azole stress responses but is critical for the initiation of filamentous growth.[213] Thus, these and other key mediators of fungal signaling networks often play dual roles in both drug resistance and virulence, making them even more promising targets for the development of novel therapeutics.

A number of other avenues are beginning to be explored to attenuate the virulence of fungal pathogens and circumvent drug resistance. Investigations in *C. albicans*, *C. neoformans*, and *Aspergillus* species have demonstrated the importance of fungal sphingolipids in virulence.[214–217] Genetic and pharmacological approaches to deplete these sphingolipids, namely, inositol phosphoryl ceramides or glucosylceramides, cause reduced pathogenicity, making them attractive targets for the development of antivirulence therapeutics.[214–217] Furthermore, farnesyltransferases contribute to virulence as well as morphogenesis and cell cycle control in fungal pathogens. *C. albicans*, *C. neoformans*, and *A. fumigatus* farnesyltransferases are essential for both virulence and cellular growth.[218–221] Initial analysis of human farnesyltransferase inhibitors developed as anticancer therapeutics, including manumycin A and tipifarnib, has revealed potent antifungal activity.[219,222] Efforts targeting other fungal virulence factors including proteinases, phospholipases, and elastases, as well as other cellular pathways that impact *C. albicans* pathogenicity such as GPI anchor biosynthesis enzymes, are underway to expand the current repertoire of antifungal agents, and they represent auspicious mechanisms to minimize detrimental host effects and the emergence of resistance elicited by conventional antifungals.[19,205]

4.3. Targeting Host Immunity

Modulating the host immune system by bolstering its capacity to resist fungal infection is another alternative therapeutic avenue to combat fungal infection (Figure 5). Development of preventative immunotherapeutics and vaccines against fungal pathogens has been explored with varying degrees of success. Making use of a shared fungal antigen present in multiple common pathogenic genera led researchers to focus on cell wall β-glucans for vaccine development.[223] Although they are poorly immunogenic, conjugation of β-glucans to diphtheria toxoids has shown great promise in mounting strong anti-β-glucan responses in murine models.[224] Conjugate β-glucan-based vaccines have demonstrated initial potential in restricting infection and prolonging survival in murine models of *Candida*, *Cryptococcus*, and *Aspergillus* infection, suggesting that they may present a valuable broad-spectrum therapeutic strategy following further development.[224–227]

The *Candida* agglutinin-like sequence (ALS) protein Als3, is a hyphal-specific glycophosphatidylinositol cell wall protein with multifunctional adhesin and invasion properties.[228,229] Als3 is highly immunogenic, eliciting robust anti-Als3 B-cell responses and T-cell responses in mice, as well as anti-Als3 antibody responses in humans.[230,231] Early analysis of a recombinant N-terminal Als3 vaccine, NDV-3A, demonstrated protective efficacy in vaginal, oral, and systemic murine models of *C. albicans* infection[230,232] (Figure 5). Subsequent progression through a phase II randomized clinical trial revealed the safety and efficacy of this vaccine in treating patients with recurrent vulvovaginal candidiasis.[233] Further interrogation of the vaccine's beneficial properties also demonstrated that NDV-3A induces *C. auris* cross-reactive antibody and T-cell responses and that vaccination with Als3 protects mice from disseminated *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* infection.[229] The success of NDV-3A to date highlights that targeting host immune responses through vaccination to combat fungal infection is a viable therapeutic strategy and should encourage further investigation of additional fungal vaccines.

5. CONCLUSION AND FUTURE OUTLOOK

A thorough understanding of the mechanistic principles governing antifungal drug resistance is fundamental for the development of more effective treatment strategies to combat current and emerging fungal threats. Despite the fact that we are still learning of new ways that *Candida* can become resistant to therapeutic treatments, advances in chemical genomic approaches in diverse fungal species have enabled powerful strategies to understand how antifungal agents exert their activity, as well as how resistance can emerge to these chemical insults.[234,235] Continued focus on the identification of new efficacious compounds will also be critical in the future in order to combat these pathogens. While there has been limited success in discovering novel antifungal classes, this is at least in part due to the fact that large-scale chemical libraries are typically biased toward targeting human biology. For example, synthetic compounds that dominate pharmaceutical company libraries are enriched in molecules with chemical properties that adopt the Lipinski rule of five,[236] guidelines that may not be ideal for antimicrobials. Consequently, compounds identified in antifungal screens often overlap with compounds that have bioactivity against human cellular targets. Expanding screens of synthetic molecules to libraries that typically have not

yielded bioactivity in human targets (so-called chemical dark matter) can offer an entry point into a selective antifungal target space.[237] Focusing on scientific methods that adopt clever screens for new targets coupled with mining of natural products and synthetic molecule libraries will provide opportunities to yield new antifungal leads. Collectively, advanced functional genomics resources, promising new screening platforms, and renewed interest from governments and the pharmaceutical industry in combatting drug-resistant infectious disease provide great hope for the accelerated development of novel antifungal therapeutics.

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Biographies

Yunjin (Rachel) Lee, B.Sc., completed her undergraduate degree in Honours Biology at McMaster University. For her senior thesis project with Dr. Ana Campos' lab, she screened a library of protein kinase inhibitors to identify novel regulators and signaling pathways underlying Hydra regeneration. This piqued her interest in the use of chemical biology strategies to unravel complex biological mechanisms. After learning about the devastating impact of fungal pathogens, she wanted to be actively involved in researching new therapeutic strategies and understanding the mechanisms of antifungal drug resistance. Rachel joined Dr. Leah Cowen's lab in December 2018 and is currently investigating the mechanisms of novel antifungal compounds that possess activity against *Candida albicans*, as well as characterizing the roles of protein kinases in *C. albicans* pathobiology.

Emily Puumala, M.Sc., completed her integrated Master's degree at Durham University's Department of Biosciences in Durham, U.K. Her research focused on the structural and functional properties of Rap, a bacteriophage lambda Holliday junction resolvase, as a member of Dr. Gary Sharples' research group. Emily has been a Ph.D student in Dr. Cowen's lab at the University of Toronto since December 2018. She is currently focused on identifying and investigating novel antifungal compounds to combat drug resistance in the emerging pathogen *Candida auris*.

Nicole Robbins, Ph.D, has been a senior research associate in Dr. Cowen's lab since July 2016. She received her undergraduate degree from McMaster University and her Ph.D. from the University of Toronto. As a doctorate student with Dr. Cowen, she focused on mechanisms by which the molecular chaperone Hsp90 governed fungal drug resistance in *Candida albicans*. Nicole then went on to pursue a postdoctoral fellowship with Dr. Gerry Wright at McMaster University, where she used high-throughput screening and chemical biology approaches to elucidate antifungal mode-of-action. As a research associate with Dr. Cowen, she is involved in many projects that aim to provide a global view of the circuitry that govern drug resistance, morphogenesis, and virulence in emerging fungal pathogens.

Leah E. Cowen, Ph.D., is Professor and Chair of the Department of Molecular Genetics at the University of Toronto and Co-founder and Chief Scientific Officer of *Bright Angel*

Therapeutics, a company that leverages state-of-the-art technologies for development of novel antifungal therapeutics. She received her undergraduate degree from the University of British Columbia and a Ph.D. from the University of Toronto, and she pursued postdoctoral studies at the Whitehead Institute, Massachusetts Institute of Technology. Her laboratory takes an interdisciplinary approach to understand what allows some microbes to exploit the host and cause disease and to develop new strategies to treat life-threatening infectious disease. Dr. Cowen has an outstanding track record of excellence in research, scholarship, and education. She has published over 85 high-impact research articles and delivered over 90 invited lectures. She has been recognized with a myriad of awards including a *Burroughs Wellcome Fund Career Award*, *Grand Challenges Canada Star in Global Health Award*, *Merck Irving S. Sigal Memorial Award*, *E.W.R. Steacie Award*, *Canada Research Chair in Microbial Genomics & Infectious Disease*, and *Fellowship of the American Academy of Microbiology*. Dr. Cowen is Co-Director of the CIFAR program *The Fungal Kingdom: Threats & Opportunities*. She has cultivated a network of international excellence through her extensive team of over 70 collaborators and is advancing knowledge translation as Chief Scientific Officer of *Bright Angel Therapeutics*.

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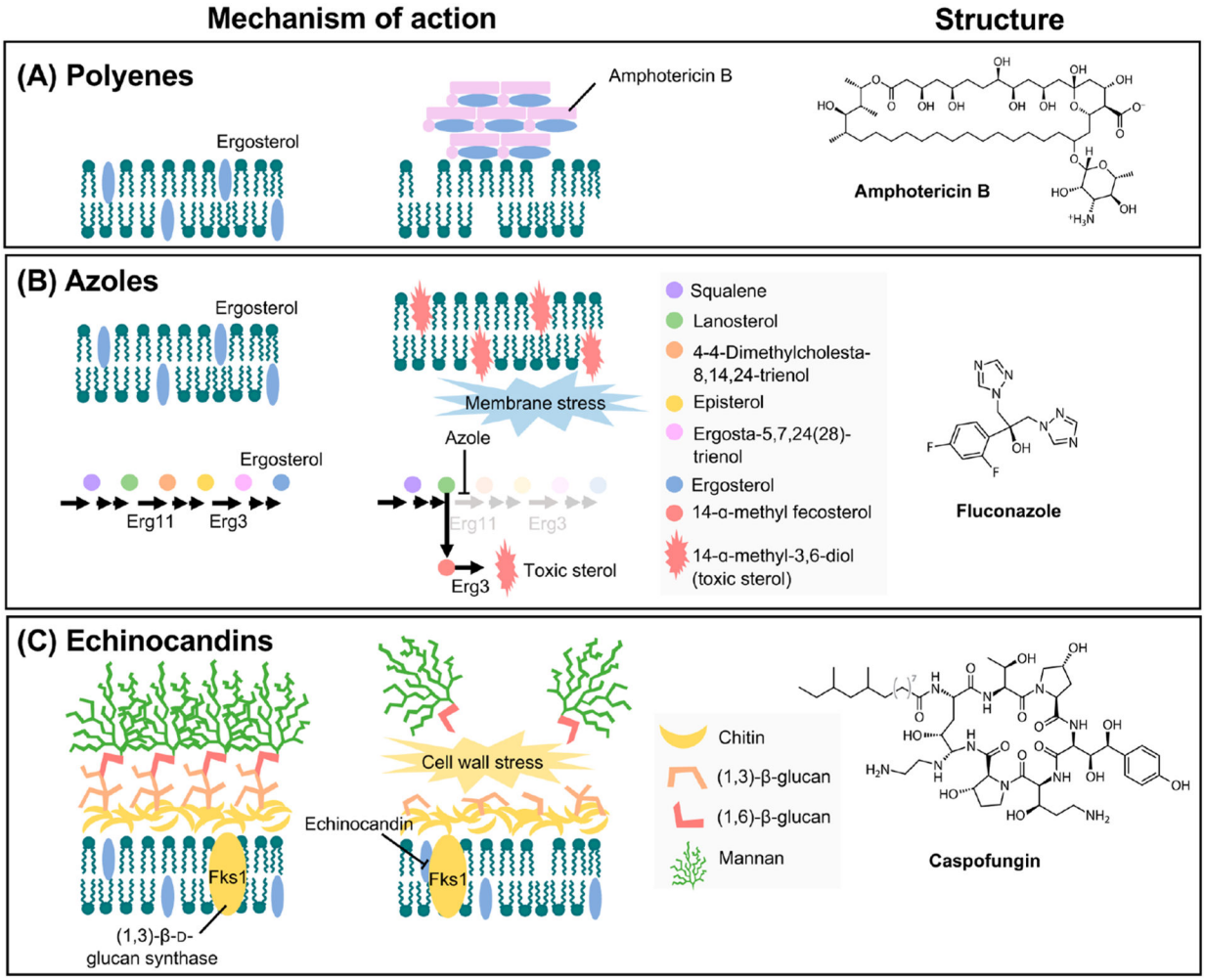

Figure 1.

Antifungal mechanisms of action and structures. (A) Polyenes, such as amphotericin B, act as a fungicidal "sterol sponge" by forming extramembranous aggregates that extract ergosterol from lipid bilayers. (B) Azoles exert fungistatic activity by inhibiting lanosterol 14-α-demethylase (encoded by *ERG11*), which leads to a block in ergosterol synthesis and the accumulation of toxic sterol intermediates, including 14-α-methyl-3,6-diol produced by Erg3. (C) Fungal cell walls are composed of (1,3)-β-D-glucan covalently linked to (1,6)-β-D-glucan, as well as chitin and mannan. Echinocandins prevent the synthesis of (1,3)-β-D-glucan by inhibiting the (1,3)-β-D-glucan synthase (encoded by *FKS1* in *C. albicans* and *C. auris* and by both *FKS1* and *FKS2* in *C. glabrata*); this results in a loss of cell wall integrity and severe cell wall stress. The structure of a representative antifungal from each class is depicted to the right of each mechanism. Adapted with permission from ref 26. Copyright 2008 Springer Nature.

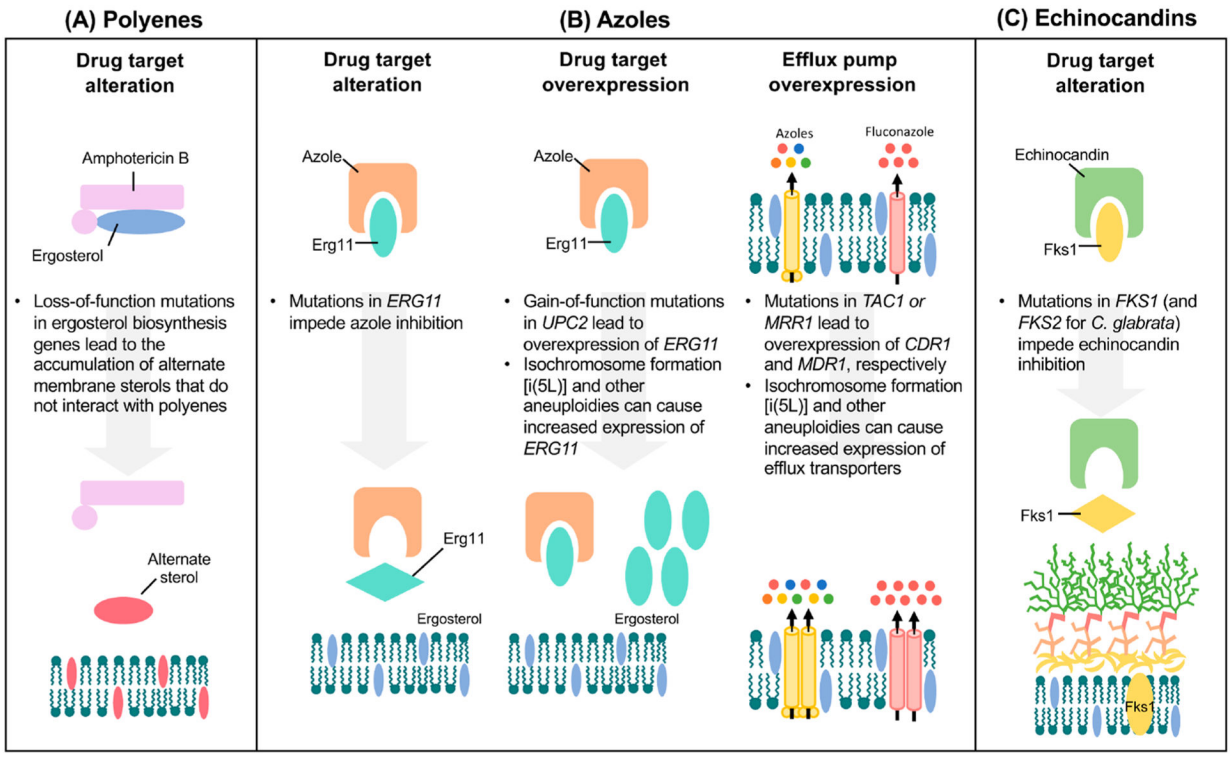

Figure 2.

Molecular mechanisms of antifungal drug resistance. (A) Resistance to polyenes is primarily mediated by the depletion of the target ergosterol through loss-of-function mutations in ergosterol biosynthesis genes. This leads to the production of alternate sterols, which do not effectively interact with polyenes and therefore are not extracted from the fungal cell membrane. (B) Resistance to azoles can occur through substitutions to the azole target, Erg11, which leads to lower drug-binding affinity for the lanosterol demethylase enzyme (left panel). Overexpression of the drug target can occur through gain-of-function mutations in the transcriptional activator, *UPC2*, or through the formation of aneuploidies, such as [i(5L)], which directly increase the copy number of *ERG11* (middle panel). Azole resistance is also acquired through the upregulation of ABC transporters (yellow), including Cdr1 and Cdr2, by activating mutations in specific transcription factors (*TAC1* in *C. albicans* and *C. auris* and *PDR1* in *C. glabrata*). Additionally, overexpression of the MF transporter (pink), Mdr1, through activating mutations in the transcriptional factor, *MRR1*, confers azole resistance (right panel). Efflux pumps can also be overexpressed through aneuploidy formation. (C) Resistance to echinocandins primarily involves mutations in *FKS* genes that encode the catalytic subunit of the drug target (1,3)-β-D-glucan synthase. For *C. albicans* and *C. auris*, mutations conferring echinocandin resistance occur in *FKS1*, while for *C. glabrata*, mutations occur in both *FKS1* and its paralogue *FKS2*.

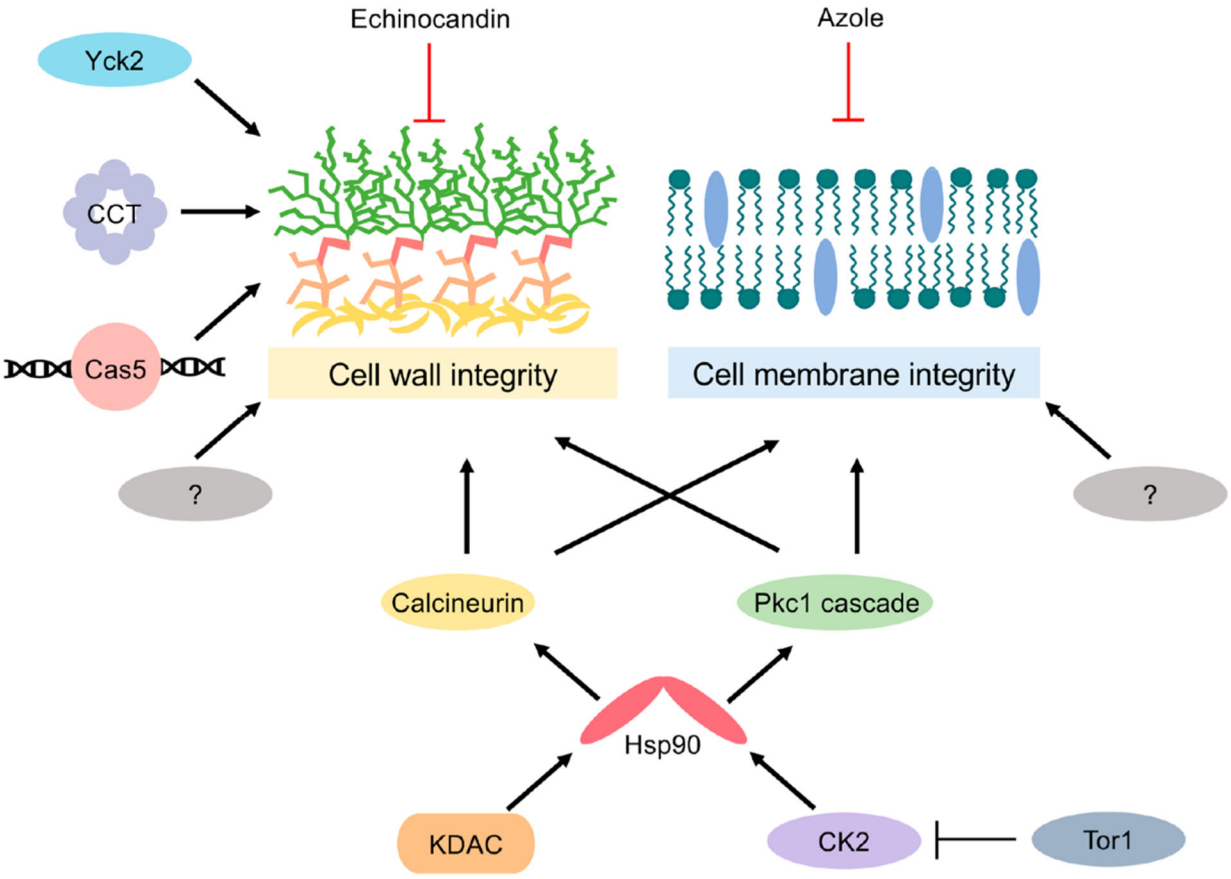

Figure 3.

Cellular stress responses important for mediating cell wall and cell membrane integrity. A global cellular regulator governing antifungal tolerance and resistance is the molecular chaperone, Hsp90. In *C. albicans*, Hsp90 is post-translationally regulated by the protein kinase complex CK2 as well as lysine deacetylases (KDACs). Key client proteins of Hsp90 important for mediating cell wall and cell membrane stress responses include the protein phosphatase calcineurin and several components of the PKC cell wall integrity pathway (Pkc1, Bck1, Mkk2, and Mkc1). Additional cellular factors important for mediating tolerance and resistance to the echinocandins include the transcription factor Cas5, the eukaryotic chaperonin containing TCP-1 (CCT) complex, and the protein kinase Yck2. Notably, many other cellular factors contribute to these cellular stress responses, with many more enigmatic regulators that remain to be identified. Perturbation of any of these signaling pathways in combination with the cell wall or cell membrane stress elicited by the echinocandins and azoles, respectively, culminates in fungal growth inhibition.

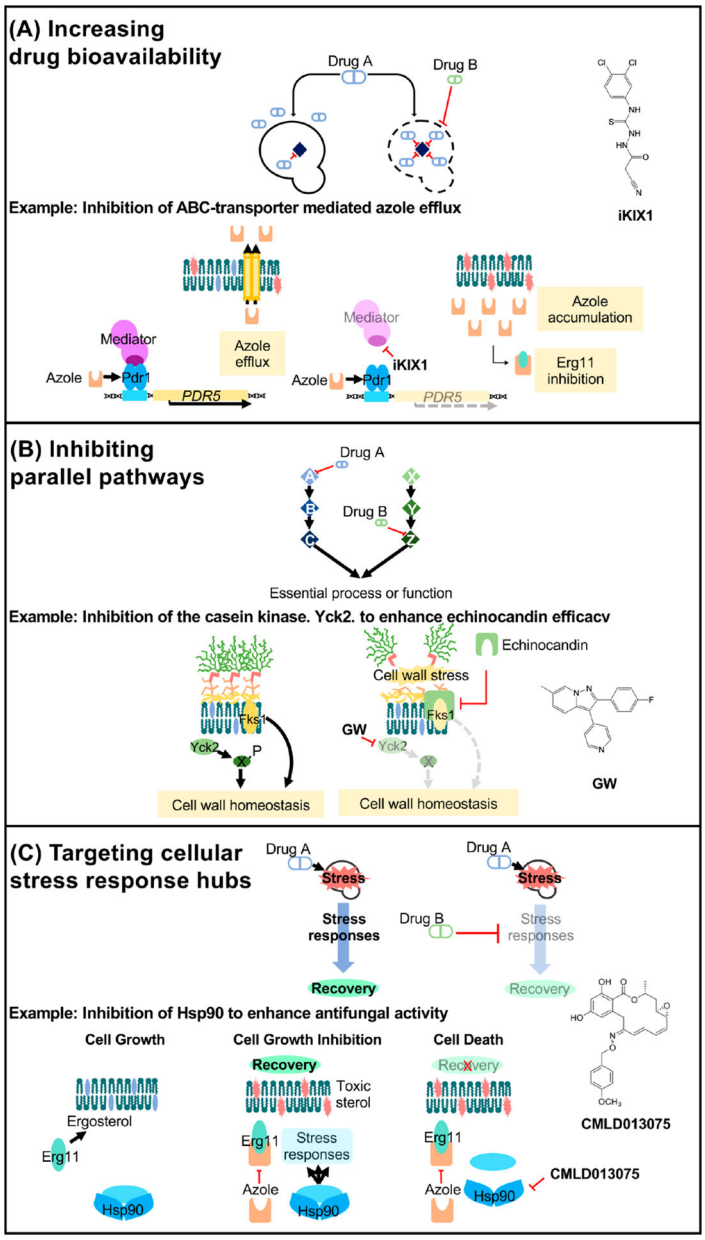

Figure 4.

Mechanisms of drug potentiation. (A) Two drugs can potentiate the activity of one another when one drug's action (drug B) increases the bioavailability of another (drug A) within the target cell. For example, in *C. glabrata*, the small-molecule iKIX1 (structure shown on right) synergizes with the azoles and resensitizes resistant isolates to fluconazole. iKIX1 disrupts the interaction between the KIX domain within the Gal11/Med15 mediator complex (dark purple) and Pdr1, which prevents upregulation of genes encoding efflux pumps (such as *PDR5*) in response to the azoles. (B) Two drugs targeting proteins of parallel pathways that converge on a single, essential process or function may also display potentiation activity. Pharmacological inhibition of the nonessential stress kinase Yck2 with the novel 2,3-aryl-pyrazolopyridine compound, GW, potentiates echinocandin efficacy in *C. albicans*

and exacerbates echinocandin-mediated cell wall disruption. (C) Fungal stress responses play a major role in basal antifungal tolerance as well as resistance. Hsp90 is a global regulator of stress-response circuitry, promoting tolerance and resistance to drug-induced stress. Key regulators of cellular stress responses (light blue) are stabilized by Hsp90 in response to antifungal treatment, enabling signal transduction that is necessary to survive in the presence of a drug-induced stress. Pharmacological compromise of Hsp90 using the fungal-selective inhibitor CMLD013075 blocks these signaling networks, thereby preventing the evolution of resistance and abrogating resistance once it has evolved.

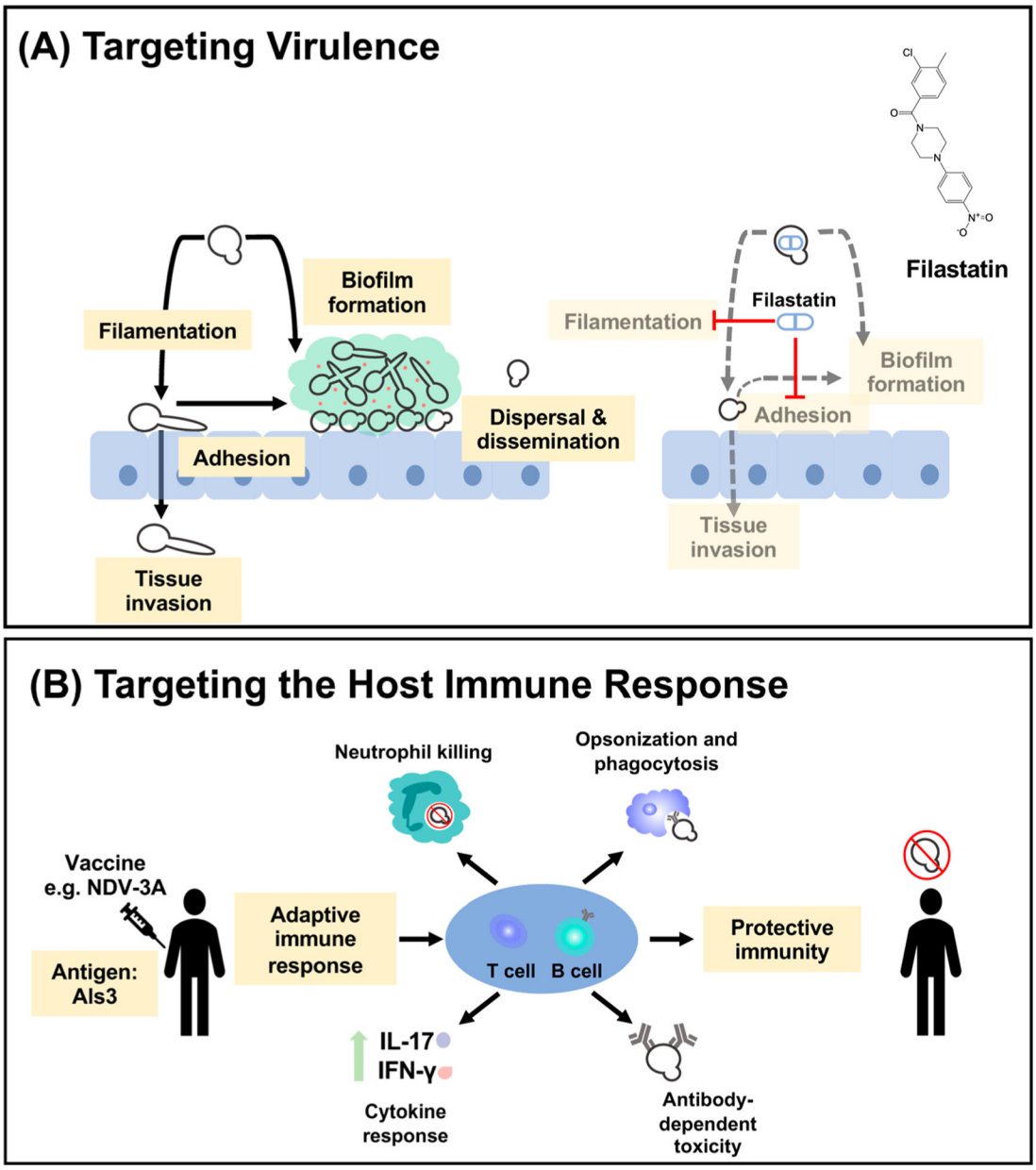

Figure 5.

Targeting fungal virulence and the host immune response as alternative antifungal strategies. (A) Thwarting fungal infection can be achieved through manipulation of key fungal virulence traits. The *C. albicans* morphogenetic transition between yeast and filamentous states enables it to adhere to, damage, invade, and disseminate through host tissues, as well as to form biofilms. The small-molecule filastatin inhibits *C. albicans* filamentation, surface adhesion, and biofilm formation. (B) Vaccination is a potential therapeutic approach to combat fungal disease, particularly in vulnerable populations. Development of the novel anti-*Candida* vaccine NDV-3A employs the highly immunogenic adhesin and invasin Als3 to elicit an adaptive immune response mediated by antibody and T-cell responses. Antibody-facilitated opsonization for phagocytosis, T-cell mediated proliferation of proinflammatory

cytokines IL-17 and IFN-γ, and enhancement of *C. albi*cans neutrophil killing are characteristic of immunization and facilitate protective immunity in the host.