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Review

The role of *Candida albicans* stress response pathways in antifungal tolerance and resistance

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SUMMARY

Human fungal pathogens are the causative agents of devastating diseases across the globe, and the increasing prevalence of drug resistance threatens to undermine the already limited treatment options. One prominent pathogen is the opportunistic fungus *Candida albicans*, which can cause both superficial and serious systemic infections in immunocompromised individuals. *C. albicans* antifungal drug resistance and antifungal tolerance are supported by diverse and expansive cellular stress response pathways. Some of the major players are the Ca^{2+}-calmodulin-activated phosphatase calcineurin, the protein kinase C cell wall integrity pathway, and the molecular chaperone heat shock protein 90. Beyond these core signal transducers, several other enzymes and transcription factors have been implicated in both tolerance and resistance. Here, we highlight some of the major stress response pathways, key advances in identifying chemical matter to inhibit these pathways, and implications for *C. albicans* persistence in the host.

INTRODUCTION

Fungal pathogens represent a major burden to human health, as they are responsible for approximately 1.6 million deaths worldwide every year (Almeida et al., 2019). These pathogens are primarily opportunistic, causing systemic infections which afflict immunocompromised populations such as those patients' undergoing chemotherapy or steroid treatments, as well as individuals suffering from AIDS (Fisher et al., 2020). One prominent pathogen is *Candida albicans*, which is a natural component of the human microbiota that can become pathogenic upon immune suppression of its host. Global invasive candidiasis cases have been estimated at 700,000 annually, with *C. albicans* classified as the leading etiological agent of these nosocomial infections (Bongomin et al., 2017). Given that these opportunistic infections are a leading co-morbidity for a range of underlying health conditions, they are associated with extremely high mortality rates of ~40% (Kullberg and Arendrup, 2015). This is compounded by the inadequate options available to treat these diseases.

Treatment of systemic fungal infections is limited to three major classes of antifungals: azoles, echinocandins, and polyenes, each of which target components of the fungal cell membrane or cell wall (Figure 1) (Lee et al., 2020; Perfect, 2017). The azoles inhibit the biosynthesis of ergosterol, which is an essential component of the fungal cell membrane, akin to cholesterol in humans (Lee et al., 2020). Specifically, azoles target lanosterol 14-α-demethylase, encoded by *ERG11* in yeasts, disrupting the production of ergosterol and resulting in an accumulation of the sterol intermediate 14-α-methyl-3,6-diol, produced by the Δ-5,6-desaturase enzyme encoded by *ERG3* (Robbins et al., 2017; Watson et al., 1989). These changes in sterol homeostasis result in perturbation of membrane stability and inhibition of fungal growth. The echinocandins target the fungal cell wall by inhibiting (1,3)-β-D-glucan synthase, encoded by *FKS1*, inducing a severe cell wall stress and leading to a loss of cell wall integrity (Denning, 2003; Robbins et al., 2017). Finally, the polyenes, like the azoles, target ergosterol; however, they do so by acting as an ergosterol sponge, forming extramembranous ergosterol aggregates through extraction of the lipid from membranes, which results in cell death (Anderson et al., 2014; Guo et al., 2021). Unfortunately, each of these antifungals suffer from major drawbacks such as poor bioavailability, limited spectrum of activity, or host toxicity (Lee et al., 2020; Robbins et al., 2017). In addition, their increased utility in clinical settings has contributed to a surge in resistant isolates, such that the Centers for Disease Control and Prevention declared azole-resistant *Candida* a

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https://doi.org/10.1016/j.isci.2022.103953

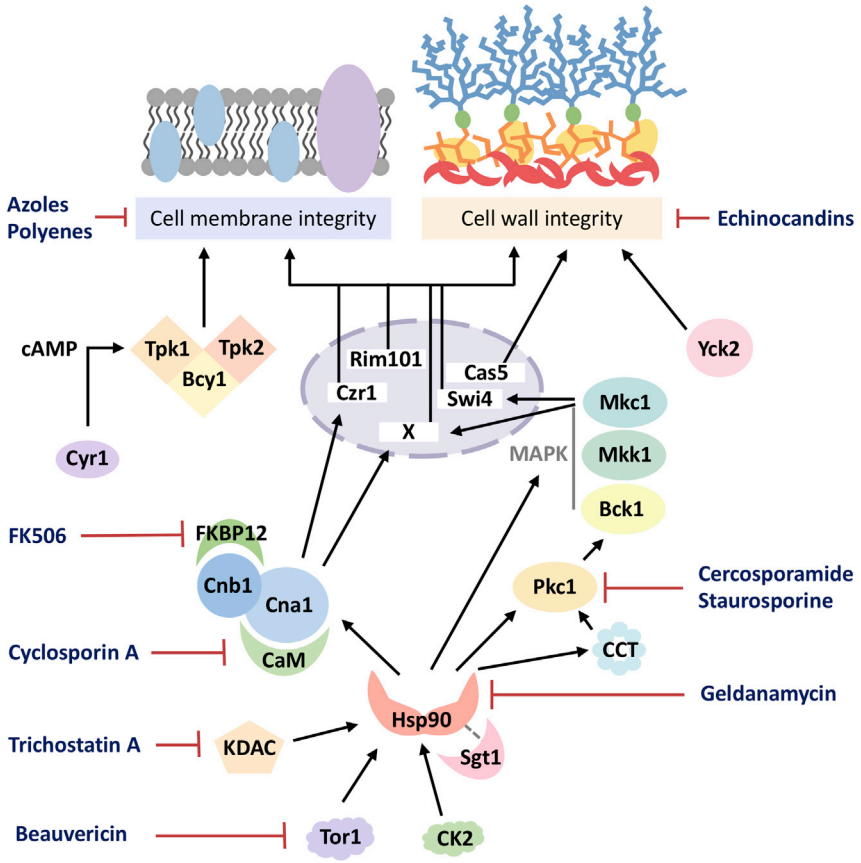

Figure 1. Cellular stress responses important for mediating cell wall and cell membrane integrity upon antifungal treatment

The azoles and polyenes target the biosynthesis of ergosterol, or ergosterol directly, leading to cell membrane stress. The echinocandins inhibit the biosynthesis of β-glucan leading to cell wall stress. A central cellular regulator modulating antifungal tolerance and resistance is the molecular chaperone Hsp90, which is post-translationally regulated by the protein kinase complex CK2 as well as lysine deacetylases (KDACs). Hsp90 also interacts with its co-chaperone Sgt1 and with the kinase Tor1. Two important client proteins of Hsp90 are the protein phosphatase calcineurin (composed of Cna1, Cnb1, and its immunophilins FKBP12 and cyclophilin A (CaM)) and components of the PKC cell wall integrity pathway (Pkc1, Bck1, Mkk1, and Mkc1). These two cascades activate the transcription factors Crz1 and Swi4, respectively, as well as additional regulators that remain to be identified. Additional cellular factors include the transcription factors Cas5 and Rim101, the eukaryotic chaperonin containing TCP-1 (CCT) complex, and the protein kinase Yck2. Finally, the cyclic AMP (cAMP) protein kinase A (composed of Tpk1, Tpk2, and Bcy1 subunits) cascade has also been implicated in drug-induced cell membrane stress. Notably, there are many other factors which contribute to the cellular response to antifungal stress that remain to be identified. Pharmacological inhibitors that target these regulators are depicted in blue font.

serious threat to public health (Centers for Disease Control and Prevention, 2019). Thus, the rise in antifungal drug resistance has applied mounting pressure on the scientific community to understand the biological mechanisms that govern the emergence and maintenance of resistance and to develop new therapeutic strategies to thwart them.

In general, fungi often exhibit either tolerance or resistance to antifungals, both of which have clinically significant implications (Berman and Krysan, 2020). Tolerance is defined as the ability of an isogenic subpopulation of drug-sensitive cells to grow, albeit slowly, above the minimum inhibitory concentration (MIC) (Rosenberg et al., 2018). In contrast, resistance is when an isolate grows above a clinically determined breakpoint MIC for a given antifungal (Berman and Krysan, 2020). Importantly, both tolerance and resistance enable fungal populations to persist within the human host, leading to therapeutic failure. It has long been established that fungal stress responses are integral to antifungal tolerance as numerous studies have employed both pharmacological and genetic techniques to demonstrate this interaction (Caplan

et al., 2018; Cowen, 2013; Cowen and Lindquist, 2005; Cruz et al., 2002; LaFayette et al., 2010; Lee et al., 2020; Shapiro et al., 2011; Shekhar-Guturja et al., 2016a; Singh et al., 2009; Xie et al., 2017). The relationship is far more condition specific when looking at drug resistance, where the specific mechanism of resistance can impact if it is stress response dependent (Cowen and Lindquist, 2005; Hill et al., 2015). Here, we outline some of the major pathways implicated in these phenomena and how they enable fungal populations to persist despite antifungal treatment (Figure 1). Finally, we highlight recent advances in structure-guided approaches to identify molecules that specifically inhibit fungal stress response networks as a promising therapeutic strategy to combat these life-threatening infections.

STRESS RESPONSE REGULATORS

Calcineurin

Calcineurin is a highly conserved Ca^{2+}-calmodulin-activated protein phosphatase, which is integral in regulating diverse physiological processes, including cell cycle progression, cation homeostasis, morphogenesis, and responses to environmental stress (Juvvadi et al., 2014). In *C. albicans*, calcineurin is formed by a heterodimer composed of a catalytic subunit, Cna1, and a regulatory subunit, Cnb1. When activated, this initiates a signaling cascade leading to the dephosphorylation and activation of the major transcription factor Crz1, as well as other factor(s) remaining to be identified (Figure 1) (Cowen et al., 2006; Singh et al., 2009). While this stress response cascade is pivotal in responding to diverse environmental perturbations, its involvement in mediating antifungal tolerance and resistance contributes to the ability of *C. albicans* to persist during infection. Despite not being essential under basal laboratory conditions in *C. albicans*, calcineurin is critical for survival in the presence of serum (Blankenship et al., 2003), a key component of human blood. Furthermore, it is essential for tolerating cell membrane or cell wall stresses elicited by azoles or echinocandins, respectively (Cruz et al., 2002; Sanglard et al., 2003; Singh et al., 2009). For example, treatment of *C. albicans* both *in vitro* and *in vivo* with the pharmacological inhibitors FK506 or cyclosporin A enhances sensitivity of *C. albicans* to the azoles and echinocandins (Cruz et al., 2002; Sanglard et al., 2003; Singh et al., 2009), and genetic deletion of *CNB1* leads to increased antifungal sensitivity (Cruz et al., 2002). Importantly, inhibition of calcineurin causes the normally fungistatic azoles to have fungicidal activity (Onyewu et al., 2003), suggesting that calcineurin is core to the fungistatic nature of ergosterol biosynthesis inhibitors. Furthermore, the paradoxical growth effect in which *C. albicans* can persist and grow upon exposure to high concentrations of echinocandins (Berman and Krysan, 2020; Wiederhold, 2007; Wiederhold et al., 2005), also requires the calcineurin pathway, as addition of cyclosporin A abolishes growth at higher drug concentrations (Wiederhold et al., 2005). Interestingly, deletion of *CRZ1*, a transcription factor and major downstream effector of calcineurin, leads to increased sensitivity to the azoles, but not to the same degree as deletion of *CNB1* (Onyewu et al., 2004; Santos and de Larrinoa, 2005). This phenomenon holds true for echinocandin tolerance, which is reduced upon deletion of *CRZ1*, but not to the same extent as *CNA1* deletion (Singh et al., 2009), suggesting that Crz1 is not the sole modulator of azole or echinocandin tolerance downstream of calcineurin in *C. albicans*. While calcineurin plays a critical role in enabling antifungal-induced stress tolerance in diverse fungal species, the importance of Crz1 orthologs is more variable (Chow et al., 2017; de Castro et al., 2019; Park et al., 2019), highlighting that different species employ alternate signaling networks to mediate this critical stress response cascade. Further exploration of alternate regulators of calcineurin signaling across diverse fungal species may illuminate important and fungal-specific biological targets that can be exploited for therapeutic intervention.

Calcineurin has also been found to play a role in drug resistance, although this is highly dependent on the mechanism by which resistance is acquired. For example, loss-of-function mutations in *ERG3* lead to azole resistance that is calcineurin dependent (Cowen and Lindquist, 2005). However, when resistance is governed by upregulation of efflux transporters, inhibition of calcineurin-mediated stress responses is no longer effective at increasing drug sensitivity (Cowen and Lindquist, 2005; Hill et al., 2015). Echinocandin resistance is primarily conferred through amino acid substitutions within hot spot regions in the drug target Fks1 (Park et al., 2005b). Combination treatment with an echinocandin and cyclosporin A against clinical isolates harboring *FKS1* mutations revealed a synergistic interaction with eight out of 14 isolates (Singh et al., 2009). Interestingly, although a subset of these clinical strains harbored identical *FKS1* mutations, differences were observed in the ability of cyclosporin A to enhance echinocandin efficacy, suggesting that additional factors influence the impact of calcineurin on echinocandin resistance that remain to be discovered.

Another key developmental state that *C. albicans* adopts to persist in the host is the formation of surface-associated communities called biofilms, which exhibit remarkable levels of drug resistance relative to their

planktonic counterparts (d'Enfert, 2006; Wall et al., 2019). Resistance is orchestrated by several factors such as altered cell wall and cell membrane composition, increased efflux pump activity, and the production of a polysaccharide matrix that acts as a physical barrier to small molecules (Mukherjee et al., 2003; Nett et al., 2010; Nett et al., 2011, d'Enfert, 2006). Remarkably, genetic or pharmacological inhibition of calcineurin acts synergistically with the azoles, which normally are ineffective against biofilms, during biofilm growth *in vitro* and *in vivo* (Uppuluri et al., 2008), highlighting the importance of this stress response network during both planktonic and biofilm growth. While other stress response pathways have been implicated in biofilm growth and drug resistance (Kumamoto, 2005; Lee et al., 2016; Robbins et al., 2011; Uppuluri et al., 2008), our understanding of the factors that contribute to drug tolerance and resistance in this developmental state remains incomplete given the lack of comprehensive functional genomic screens performed under these conditions.

Protein kinase C

The protein kinase C (PKC) cell wall integrity pathway is critical to cell wall homeostasis, morphogenesis, and responses to cell wall and cell membrane stress (LaFayette et al., 2010; Reinoso-Martin et al., 2003; Xie et al., 2016). Pkc1 is a core kinase that acts upstream of a MAPK signaling cascade consisting of the sequential kinases Bck1, Mkk1, and Mkc1, which in turn activate several transcription factors to initiate a gene expression program pivotal for responding to cellular stress (Figure 1) (Shapiro et al., 2011). The PKC cascade in conjunction with calcineurin upregulates chitin synthesis, which is integral to maintaining cell wall integrity. Another pathway functionally related to the PKC pathway is the high osmolarity pathway (HOG), which also uses a MAPK cascade to maintain cell wall integrity and regulate chitin synthesis (Munro et al., 2007). In fact, while echinocandin treatment reduces cell wall β-glucan levels by over 70%, there is a nearly 900% compensatory increase in cell wall chitin (Stevens et al., 2006), which is dependent on core components of the PKC, calcineurin, and HOG pathways (Munro et al., 2007) (Munro et al., 2007). While Pkc1 is not essential in *C. albicans* under basal conditions, a *PKC1* homozygous deletion mutant is hypersensitive to the echinocandins (Reinoso-Martin et al., 2003) and the azoles (LaFayette et al., 2010). Pkc1 regulates azole and echinocandin tolerance via the MAPK cascade as deletion of the terminal kinase *MKC1* also enhances sensitivity to these antifungals (Caplan et al., 2018; LaFayette et al., 2010). However, while deletion of *PKC1* renders the azoles fungicidal, homozygous deletion of *MKC1* does not, suggesting that Pkc1 has other downstream targets important for responding to cell membrane stress (LaFayette et al., 2010). The PKC pathway has also been implicated in *C. albicans* paradoxical growth in the echinocandins, as deletion of *MKC1* abolishes growth at high concentrations (Wiederhold et al., 2005).

In addition to drug tolerance, the PKC pathway has an important role in drug resistance. Two structurally unrelated Pkc1 inhibitors, cercosporamide and staurosporine, reduce azole resistance in selected early clinical isolates that were obtained from a patient with HIV undergoing fluconazole therapy (LaFayette et al., 2010; White, 1997). Pkc1 inhibition also abrogates azole resistance in a *C. albicans* strain with loss-of-function mutations in *ERG3* (LaFayette et al., 2010). With respect to echinocandin resistance, genetic depletion of *PKC1* or deletion of *MKC1* in a clinical isolate containing a mutation in *FKS1* abrogates the resistance phenotype (Caplan et al., 2018). Finally, the PKC pathway has important functions in biofilm drug resistance, as Mkc1 is required for biofilm formation (Kumamoto, 2005), and mutants with disruption of downstream pathway components, including the cell wall biosynthesis protein Smi1 and the transcription factor Rlm1, are deficient in biofilm matrix production and more susceptible to the azoles (Nett et al., 2011).

Heat shock protein 90

Heat shock protein 90 (Hsp90) is an essential molecular chaperone whose client proteins in the model yeast *Saccharomyces cerevisiae* represent over 10% of the proteosome (Zhao et al., 2005). Its stabilization and regulation of those factors that function as key cellular signaling regulators place Hsp90 as a critical hub of stress response networks (O'Meara et al., 2017; Taipale et al., 2010). Under planktonic conditions, Hsp90 stabilizes the catalytic subunit of calcineurin and core components of the PKC pathway, including Pkc1, Bck1, and Mkc1 (Figure 1) (Caplan et al., 2018; LaFayette et al., 2010; Singh et al., 2009). Therefore, Hsp90 is integral to azole and echinocandin tolerance and resistance, as pharmacological inhibition or genetic depletion increases drug sensitivity (LaFayette et al., 2010; Singh et al., 2009). Specifically, compromising Hsp90 pharmacologically with the inhibitor geldanamycin phenocopies both calcineurin and PKC inhibition (LaFayette et al., 2010; Singh et al., 2009). Indeed, when a series of azole-resistant clinical isolates (White, 1997) and experimentally evolved strains (Cowen and Lindquist, 2005) were assessed, geldanamycin was able to abrogate resistance unless there was heightened drug efflux (Cowen and Lindquist, 2005; Hill et al., 2015; LaFayette et al., 2010).

As a key regulator of stress signaling, Hsp90 has also been implicated in the cellular response to amphotericin B. Resistance to this polyene is often achieved through alterations in the ergosterol biosynthesis pathway, which lead to severe fitness defects. Therefore, these cells rely heavily on Hsp90, and its client proteins calcineurin, Pkc1, and Hog1 for survival even in the absence of stress (Vincent et al., 2013). Furthermore, Mycograb, a recombinant antibody against fungal Hsp90, demonstrates a synergistic interaction with amphotericin B against a wide variety of *Candida* species both *in vitro* and in mouse models of infection (Matthews et al., 2003). However, given that it was proposed that this antibody functioned at the cell surface where Hsp90 is not known to reside, subsequent analyses suggested these antifungal effects were due to an off-target mechanism that remains to be fully understood (Richie et al., 2012).

Consistent with the stress response pathways it modulates, Hsp90 is critical to *C. albicans* biofilm formation, with genetic depletion leading to impaired growth and dispersal *in vitro* (Robbins et al., 2011). Impairment of Hsp90 also abrogates azole resistance in biofilms *in vitro* and *in vivo*. Interestingly, this was calcineurin and Mkc1 independent as depletion of Hsp90 did not lead to destabilization of these regulators, suggesting alternative client proteins that are important in this distinct growth state remain to be identified (Robbins et al., 2011).

Several additional proteins that interact with Hsp90 have also been implicated in drug tolerance and resistance (Figure 1). The Hsp90 co-chaperone Sgt1 is important for regulating antifungal-induced stress, as genetic depletion of *SGT1* abrogates basal tolerance and acquired resistance to azoles due at least in part to impairment of calcineurin signaling (Shapiro et al., 2012). Additionally, the eukaryotic chaperonin containing TCP-1 (CCT) complex, which associates with Hsp90, is implicated in regulating echinocandin tolerance and resistance via activation of the PKC signaling cascade (Caplan et al., 2018). An additional protein kinase with roles in governing azole susceptibility is casein kinase 2 (CK2), as overexpression of the catalytic subunit of the CK2 complex decreases the efficacy of fluconazole. This decrease in susceptibility was dependent on calcineurin (Bruno and Mitchell, 2005), which is consistent with the observation that CK2 phosphorylates Hsp90 to regulate its chaperoning activity (Diezmann et al., 2012; Mollapour et al., 2011). Finally, lysine deacetylases (KDACs) also serve as key regulators of azole tolerance and resistance at least in part through post-translational modification of Hsp90. While functionally redundant, the KDACs Hos2, Hda1, Rpd3, and Rpd31 all regulate Hsp90 function in *C. albicans*, and therefore, mediate critical responses upon azole exposure (Li et al., 2017). Pharmacological inhibition of KDACs with trichostatin A phenocopies inhibition of Hsp90 and abrogates Hsp90-dependent azole resistance due to the importance of acetylation in controlling Hsp90's interaction with calcineurin (Robbins et al., 2012). While the mechanisms are not fully understood, KDAC inhibitors have also been reported to enhance sensitivity of *C. albicans* to the azoles through the upregulation of ergosterol biosynthesis and efflux genes (Smith and Edlind, 2002). Despite all these examples, Hsp90 serves as a critical hub of stress response networks and therefore our understanding of the manner by which this chaperone regulates antifungal tolerance and resistance is far from saturation.

Other stress response pathways

Beyond the core stress response pathways highlighted above, there are numerous other pathways and signaling proteins involved in facilitating the emergence and maintenance of drug tolerance and resistance (Figure 1). One such example is the cyclic AMP-protein kinase A (cAMP-PKA) pathway. PKA is composed of two catalytic subunits, Tpk1 and Tpk2, as well as a regulatory subunit Bcy1, which are activated via cAMP produced by Cyr1 and its associated protein Srv2 (Shapiro et al., 2011). The cAMP-PKA pathway is important for *C. albicans* morphogenesis and the sensing of environmental cues, and therefore, is often implicated in fungal virulence (Lin and Chen, 2018; Park et al., 2005a; Sonneborn et al., 2000). Moreover, studies have specifically highlighted a role for Tpk2 and Cyr1 in regulating drug tolerance at least in part through the regulation of efflux pump expression (Jain et al., 2003; Lin and Chen, 2018). Recently, the importance of this signaling cascade in governing antifungal susceptibility was also described for the closely related, and notoriously drug-resistant, fungal pathogen *Candida auris* (Kim et al., 2021).

Other kinases have been implicated in drug susceptibility. For example, the target of rapamycin (TOR) kinase is involved in sensing changes in nutrient availability to then dictate growth and metabolism. Hyperactivation of TOR signaling in fungal pathogens reduces azole efficacy in a manner that is dependent on Hsp90's stabilization of calcineurin (Khandelwal et al., 2018). Complimentarily, chemical inhibition of TOR signaling with rapamycin abrogates *erg3*-mediated azole resistance (Robbins et al., 2010). Additionally, the natural product beauvericin, which inhibits both multidrug transporters and the kinase Tor1, potentiates azoles against *C. albicans*, blocks the emergence of drug resistance, and renders

antifungal-resistant pathogens responsive to treatment in mammalian infection models (Shekhar-Guturja et al., 2016a, 2016b). Another kinase implicated in drug resistance in the non-essential casein kinase 1 family member, Yck2. A chemical screen of kinase inhibitors led to the discovery of an inhibitor of Yck2, which abrogated echinocandin resistance *in vitro* against a *C. albicans* strain harboring an *FKS1* mutation. This was hypothesized to be due to the involvement of Yck2 in responding to cell wall stress, with inhibitor treatment or genetic depletion leading to an upregulation of cell wall proteins and damage genes (Caplan et al., 2020). Finally, in addition to the role of the HOG MAPK cascade in upregulating chitin synthesis upon exposure to cell wall stress, Hog1 also plays important roles on governing responses to amphotericin B in both *C. albicans* and *C. auris* (Guirao-Abad et al., 2020; Shivarathri et al., 2020).

Genetic screening of transcription factor mutants in *C. albicans* identified Cas5 as being involved in echinocandin tolerance (Bruno et al., 2006). Subsequent studies found that deletion of *CAS5* also abolished Fks1-mediated echinocandin resistance (Xie et al., 2017). Using genome-wide approaches, it was determined that Cas5 plays a role in coupling cell cycle dynamics to cell wall stress responses, as this transcription factor regulates distinct gene expression programs under basal and stress conditions (Xie et al., 2017). Another transcription factor that has been implicated in antifungal tolerance and resistance is the terminal transcription factor in the pH-sensing Rim pathway, Rim101. Once activated, Rim101 regulates expression of several target genes involved in growth, iron metabolism, cell wall structure, morphogenesis, adhesion, and biofilm formation. Deletion of *RIM101* results in hypersensitivity to the azoles and echinocandins (Cornet et al., 2006), and more recently the entire Rim pathway has been associated with azole and echinocandin tolerance with deletion of any component leading to hypersensitivity (Garnaud et al., 2018).

Finally, enzymes involved in post-translational modifications are also involved in cellular stress responses (Lee et al., 2020). An example of such modifiers is lysine acetyltransferases (KATs) that modulate azole susceptibility. Genetic impairment of a component of the *C. albicans* Spt-Ada-Gcn5-acetyl-transferase (SAGA) coactivator complex, *ADA2*, confers hypersensitivity to fluconazole due to impaired upregulation of efflux (Sellam et al., 2009). Ada2 was also identified as playing a role in caspofungin tolerance (Bruno et al., 2006), highlighting the SAGA complex regulates tolerance to diverse cellular stressors.

While these examples highlight the complexity of the signaling networks that contribute to antifungal tolerance and resistance, it is important to note that comprehensive functional genomic resources in *C. albicans* remain limited. As these genomic tools continue to be developed (Fu et al., 2021), additional regulators of these stress responses will undoubtedly be uncovered.

STRESS RESPONSE PATHWAYS AS AN ANTIFUNGAL TARGET

The pivotal role of stress response pathways in fungal tolerance of environmental, antifungal, and host assault is undeniable. Therefore, effort has been placed in identifying inhibitors that specifically target these critical pathways, as they could work either alone or in combination with existing antifungals to enhance potency and reduce the likelihood of fungal pathogens persisting in a human host. Recently, advances have been made through structure-guided design of compounds targeting fungal stress response proteins. Compounds that inhibit calcineurin, such as FK506, are immunosuppressive, which creates a need for developing fungal-selective inhibitors (Juvvadi et al., 2014). Fortunately, researchers solved the crystal structure of the two subunits of calcineurin with the inhibitor FK506 and its binding protein FKBP12 in four prominent fungal pathogens. Through subsequent analysis, they identified a specific residue that was only present in fungal FKBP12, and was critical for FK506-mediated inhibition of calcineurin (Juvvadi et al., 2019). This led to the design of APX879, an FK506 analog with an acetohydrazine substitution, which exhibited a 70-fold reduction in immunosuppressive effects while retaining antifungal activity against *C. albicans* in the presence of serum or fluconazole (Juvvadi et al., 2019), conditions where calcineurin function is critical for survival (Blankenship et al., 2003; Cruz et al., 2002). Further, this molecule displayed *in vivo* efficacy alone and in combination with fluconazole against the distantly related fungal pathogen *Cryptococcus neoformans* (Juvvadi et al., 2019). Subsequent structural, genetic, and biophysical approaches suggested additional modifications of APX879 that would lead to enhanced fungal selectivity (Gobeil et al., 2021). Other groups have also developed fungal-selective calcineurin inhibitors through screening FK506 analogs to identify compounds that synergize with fluconazole to clear murine fungal infections (Lee et al., 2018).

Hsp90 is essential in all fungi and is also critical to virulence and drug-resistance mechanisms. Thus, fungal-selective inhibition of its function is expected to show broad-spectrum single-agent activity as well as

prevent/reverse resistance to echinocandins and azoles. The ability to employ Hsp90 inhibitors in combination with current antifungals represents a promising therapeutic strategy, as it has the potential to enhance efficacy, lower dosing, and limit resistance, a strategy that is commonly utilized with antibacterials and antimalarials (Spitzer et al., 2017). Structure-guided design approaches have also been applied to optimize fungal-selective Hsp90 inhibitors. Recently, a Hsp90 inhibitor that was 25-fold selective for *C. albicans* Hsp90 over the human ortholog was identified (Whitesell et al., 2019). Further synthetic efforts generated aminopyrazole-substituted resorcylate amides with over 30-fold selectivity toward *C. neoformans* Hsp90 compared to the human ortholog (Huang et al., 2020).

While significant advances have been made in generating molecules that display fungal selectivity against these conserved stress response regulators, additional barriers remain that need to be overcome. Achieving adequate fungal permeability and intracellular accumulation is key in order for fungal-selective calcineurin and Hsp90 inhibitors to become a viable option for the treatment of systemic fungal infections. This will involve the generation of molecules capable of crossing the fungal cell wall and cell membrane as well as circumventing efflux pumps that actively pump molecules out of the cell. Fortunately, such efforts are being actively pursued.

CONCLUDING THOUGHTS

Together, the importance of stress response pathways in drug resistance and tolerance is exquisitely clear. These pathways directly impact the ability of fungi to persist in their environment, and for fungal pathogens of humans, these signaling networks are key contributors to treatment failure. Thus, there is great therapeutic potential of pairing compounds inhibiting these pathways with existing antifungals. The continued exploration of the complex network of fungal stress pathways in diverse fungal pathogens will undoubtedly advance our ability to develop additional fungal-selective molecules that perturb these pathways. The exciting work to date suggests that stress response inhibitors will one day reach the clinic to assist in the eradication of persistent systemic fungal infections.

ACKNOWLEDGMENTS

We thank all members of the Cowen lab for helpful discussions. L.E.C. is supported by the Canadian Institutes of Health Research Foundation Grant (FDN-154288) and National Institutes of Health NIAID R01 Grants (R01AI127375 and R01AI120958); L.E.C. is a Canada Research Chair (Tier 1) in Microbial Genomics & Infectious Disease and co-Director of the CIFAR Fungal Kingdom: Threats & Opportunities program.

AUTHOR CONTRIBUTIONS

KRI, NM, and LEC wrote the perspective.

DECLARATION OF INTERESTS

L.E.C. is a co-founder and shareholder in Bright Angel Therapeutics, a platform company for development of novel antifungal therapeutics.

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