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Mini-Review

Candida albicans drug resistance – another way to cope with stress

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There are relatively few classes of antifungal drugs. This restricts clinicians' therapeutic choices and these choices are further reduced by the emergence of drug resistance. Exposure to antifungal drugs represents an environmental stress for the fungal pathogen *Candida albicans*. The immediate response of *C. albicans* to antifungals may be drug tolerance, which can lead to drug resistance. This article examines *C. albicans* drug resistance from the perspective of it being a stress response and investigates how commonality with other stress-response pathways gives insights into the prospects for overcoming, or preventing, drug resistance.

Introduction

Antimicrobial drug resistance is an important biological phenomenon that has a considerable impact on animal and human health. The prevalence of clinical drug resistance has increased in recent decades with the greater use, and abuse, of otherwise efficacious antimicrobial agents. This has been a significant problem for bacterial pathogens, where resistance to multiple antibiotics severely limits therapeutic options. Antimicrobial resistance is not restricted to bacteria, however, and in the 1990s fluconazole treatment failure emerged due to the development of resistance by the fungal pathogen *Candida albicans* (White *et al.*, 1998). Antifungal resistance is particularly problematic as initial diagnosis of systemic fungal infection can be delayed and there are few antifungal drugs available. The development of antimicrobial drug resistance is not a new phenomenon – micro-organisms have been responding to toxic environmental stresses for millennia (Wright, 2007). Indeed it is likely that the mechanisms utilized to confer resistance to 'novel' synthetic drugs have been selected from an extensive repertoire that has enabled micro-organisms to survive for so long in changing environments.

Exposure to antifungal drugs is, for *C. albicans*, another environmental stress that stimulates responses to mitigate the harmful effects of the drugs and permit continued growth. The antifungal stress is transduced through signalling pathways that induce stress responses and affect the growth and virulence of the organism. Stress-response signalling and its interrelation with *C. albicans* morphogenesis and virulence are complex and beyond the scope of this mini-review. Signalling pathways, and their role in virulence, have been excellently reviewed by Quinn & Brown (2007), Monge *et al.* (2006) and Roman *et al.* (2007). Although little is known about stress responses in non-*albicans Candida* species, the stress-response pathways in the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been well studied (Roman *et al.*, 2007). Despite extensive congruence in the fungal stress-response networks, there are significant differences in the stress responses of *C. albicans* (Enjalbert *et al.*, 2006). Therefore, although major differences in the stress responses of other fungi will be noted, this review will focus specifically on how *C. albicans* responds to antifungal stress and how the development of resistance is a component of the stress-response network of *C. albicans*.

Abbreviations: 5-FC, 5-fluorocytosine; ABC, ATP-binding cassette; CsA, cyclosporin A; Cyp1p, cyclophilin A; HOG, high-osmolarity glycerol; Hsp90, heat-shock protein 90; MAPK, mitogen-activated protein kinase; MFS, major facilitator superfamily; PKA, protein kinase A.

Antifungal drug targets in *C. albicans*

There are four main antifungal drug targets in *C. albicans* (Fig. 1a). The fluorinated pyrimidine analogue 5-fluorocytosine (5-FC) causes aberrant RNA synthesis and interferes with DNA replication (Akins, 2005; Sanglard & Bille, 2002). A significant proportion of *C. albicans* isolates are resistant to 5-FC, limiting its utility. The polyenes, such as amphotericin B and nystatin, are heterocyclic amphipathic molecules that insert into lipid bilayers, bind to sterols, and aggregate in annuli to form pores. These pores disrupt plasma membrane integrity and permit the efflux of cations such as K^+, which is fungicidal for *C. albicans*. Polyenes are also thought to cause oxidative damage (Sanglard & Bille, 2002). The azole antifungals, such as the triazole fluconazole, interfere with sterol biosynthesis (Fig. 1a). They inhibit the cytochrome P_{450} 14α-lanosterol demethylase, encoded by the *ERG11* gene, which is part of the ergosterol biosynthetic pathway. Inhibition of Erg11p

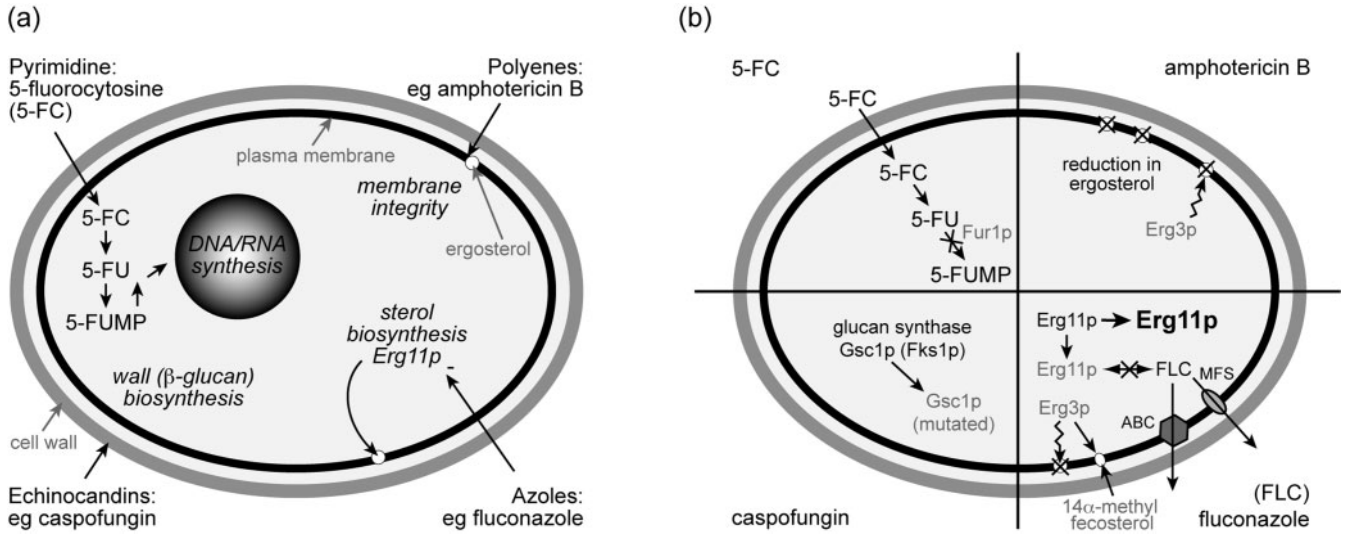

Fig. 1. (a) Targets of current antifungal drugs in *C. albicans*. (b) Mechanisms of resistance to antifungal drugs in *C. albicans*. Grey lettering indicates mutated proteins; bold lettering indicates protein overexpression. 5-FU, 5-fluorouracil; 5-FUMP, 5-fluorouridine monophosphate.

depletes the ergosterol content of membranes and results in the accumulation of toxic sterol pathway intermediates which inhibit growth (Akins, 2005; Sanglard & Bille, 2002). Azoles are thus usually fungistatic for *C. albicans*. The most recently developed class of antifungals are the cyclic lipopeptides the echinocandins. These were originally obtained from soil fungi, but semi-synthetic derivatives have been developed such as caspofungin, micafungin and anidulafungin. These drugs are thought to interfere with wall biosynthesis by inhibiting (1,3)-D-β-glucan synthase (Fig. 1a), and are fungicidal for yeasts such as *C. albicans*. Thus the current antifungal drugs that are effective against *C. albicans* primarily affect cell wall and cell membrane composition and integrity and thereby induce stress responses.

Stress response in *C. albicans*

C. albicans can be isolated from a number of environments, but is usually found in association with mammals, particularly humans. It can colonize a variety of ecological niches on humans (Cannon & Chaffin, 1999) and will experience a range of environments that will cause physiological stress. These stresses include temperature changes, ionic stress, changes in osmolarity, and oxidative stress such as that experienced in the phagosomes of neutrophils. Stresses are transduced through a variety of membrane receptors but elicit responses through conserved signalling pathways, one of the most important being the MAP kinase (MAPK) signal transduction network (Monge *et al.*, 2006). Although individual components of these pathways are conserved between fungal species there are some distinct features of the *C. albicans* stress-response network. *C. albicans* has a smaller set of core stress responses than *S. cerevisiae* or *S. pombe*, and is relatively resistant to oxidative and heat stress (Enjalbert *et al.*, 2006). *C. albicans* has parallel stress-response pathways (Fig. 2). The divergence of their regulation from that in other fungi, and *C. albicans*' specialized stress responses, may be due to the different types and amounts of stresses experienced on and within the human host (Quinn & Brown, 2007). Two important components of the MAPK network are the high-osmolarity glycerol (HOG; Hog1p) pathway and the protein kinase C

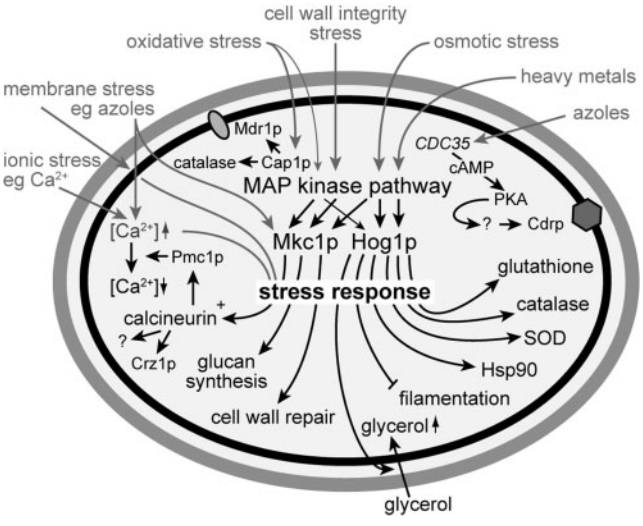

Fig. 2. Stress-response network in *C. albicans*. Grey lines indicate stresses; black lines indicate stress responses. PKA, protein kinase A (*TPK2*/*TPK1*).

cell wall integrity (Mkc1p) pathway (Fig. 2), which respond to oxidative, osmotic and heavy metal stress (Quinn & Brown, 2007; Roman *et al.*, 2007). The response to oxidative stress can be transduced via both Mkc1p and Hog1p and involves increased expression of antioxidants such as catalase, superoxide dismutase and glutathione (Fig. 2). The cell wall integrity pathway regulates glucan synthesis and cell wall repair (Navarro-Garcia *et al.*, 1998), which can be protective in response to echinocandins. Despite the involvement of the MAPK pathway, oxidative stress induces responses mainly via transcription factor Cap1p. Cap1p is also involved in multidrug resistance. Activation of Cap1p by a C-terminal truncation of the protein results in upregulation of the major facilitator superfamily (MFS) transporter gene *MDR1* (Alarco & Raymond, 1999). Hog1p is mainly responsible for the activation of core stress-response genes that are induced by osmotic or heavy metal stress. Osmotic stress results in glycerol uptake and synthesis, and exposure to heavy metal ions results in upregulation of genes involved in glutathione synthesis (Enjalbert *et al.*, 2006). Although there are similarities in the responses of *Candida glabrata* and *S. cerevisiae* to osmotic stress, *C. glabrata* lacks a HOG signalling branch, and the *C. glabrata* HOG pathway also responds to weak acid stress (Gregori *et al.*, 2007). In *C. albicans*, Hog1p represses filamentation through the *CEK1* signalling pathway (Monge *et al.*, 2006), although the formation of hyphae can be an oxidative-stress response.

The cAMP-PKA (protein kinase A) signal transduction pathway is involved in *C. albicans*' response to antifungal stress (Fig. 2); adenylate cyclase (*CDC35*) mutants no longer respond to azoles with upregulation of *CDR1* (Jain *et al.*, 2003). Azoles also cause a membrane stress that induces Mkc1p expression (J. Pla, personal communication). Membrane stress can also mediate a response through calmodulin (Fig. 2). Interestingly, exposure of cells to the heavy metal Cd^{2+} induces expression of heat-shock protein 90 (Hsp90), which stabilizes a number of proteins, including calcineurin.

The serine/threonine protein phosphatase calcineurin is highly conserved in eukaryotes and is activated in response to several stresses. It has important physiological roles in *C. albicans,* and is essential for survival during membrane stress (Cruz *et al.*, 2002). *C. albicans* calcineurin is a heterodimer composed of a catalytic subunit A (encoded by *CMP1* [*CNA1*]) and a regulatory subunit B (encoded by *CNB1*) (Fig. 3). The phosphatase activity of calcineurin is activated when it binds calmodulin in the presence of calcium ions and it affects gene expression via transcriptional regulators such as Crz1p. Calcineurin mediates tolerance to a variety of stresses, including salt and high pH in addition to membrane stress (Steinbach *et al.*, 2007) (Fig. 2). Calcineurin is essential for survival in serum (Bader *et al.*, 2006) and it is the calcium component of serum that is toxic for *C. albicans* in the absence of functional calcineurin (Blankenship & Heitman, 2005). Calcineurin also plays an important role in the tolerance of *C. albicans* to certain antifungal drugs (see below).

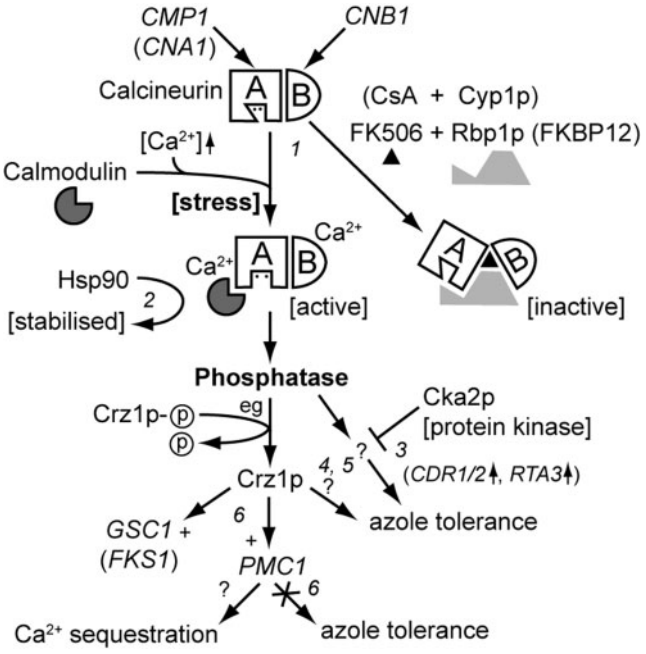

Fig. 3. Involvement of calcineurin in the azole tolerance of *C. albicans*. Dots on calcineurin subunit A represent active-site residues. CsA, cyclosporin A; Cyp1p, cyclophilin A. *1*, Fox & Heitman (2002); *2*, Cowen & Lindquist (2005); *3*, Bruno & Mitchell (2005); *4*, Karababa *et al.* (2005); *5*, Onyewu *et al.* (2005); *6*, Sanglard *et al.* (2003).

Antifungal drug resistance

The mechanisms by which *C. albicans* can become resistant to antifungal drugs have been reviewed by Sanglard & Bille (2002) and Akins (2005) and are summarized in Fig. 1(b). Resistance of clinical isolates to 5-FC most often correlates with mutations in the enzyme uracil phosphoribosyltransferase (Fur1p) that prevent the conversion of 5-fluorouracil to 5-fluorouridine monophosphate. Resistance of *C. albicans* to polyenes is rare and can be caused by a reduction in the amount of plasma membrane ergosterol, to which polyenes bind (Fig. 1b). Mutations in *ERG3*, which lower the concentration of ergosterol in the membrane, cause amphotericin B resistance and also confer resistance to azoles.

There are multiple mechanisms that can give rise to azole resistance in *C. albicans* (Fig. 1b). The drug target, Erg11p, can be overexpressed or can develop point mutations that reduce fluconazole binding (Akins, 2005; Sanglard & Bille, 2002; White *et al.*, 1998). Azole-induced *C. albicans* growth inhibition is caused by reduction in the ergosterol content of membranes, and also by the accumulation of toxic ergosterol precursors such as 14α-methylergosta-8,24(28)-dien-3β,6α-diol. If Erg3p is inactivated by mutation, in the

presence of fluconazole these cells accumulate the non-toxic sterol 14α-methylfecosterol. High-level azole resistance correlates with overexpression in the plasma membrane of the MFS drug pump Mdr1p, or the ATP-binding cassette (ABC) pumps Cdr1p or Cdr2p (Perea *et al.*, 2001; White *et al.*, 1998). These proteins have been found to pump a variety of substrates, including the azoles (Lamping *et al.*, 2007), but they do not pump the echinocandins (Niimi *et al.*, 2006). Little clinical resistance to the echinocandins has been reported so far. This may reflect its limited use to date, or that resistance events are rare. Echinocandin-resistant *C. albicans* isolates have point mutations in (1,3)-β-glucan synthase subunit Gsc1p (Baixench *et al.*, 2007).

One way to discover antifungal drug resistance mechanisms is to compare resistant clinical isolates with their susceptible parents (Perea *et al.*, 2001; White, 1997). Another way is to select for resistance *in vitro* (Albertson *et al.*, 1996; Anderson, 2005; Cowen *et al.*, 2000). These studies have shown distinct patterns in the type and combinations of mechanisms that evolve in *C. albicans* populations to confer azole resistance. Often the patterns of mechanisms that evolve mimic those seen in clinical isolates, described above, validating this approach (Anderson, 2005). Interestingly, there is often a fitness gain associated with the development of azole resistance and so resistant isolates may persist in the population after the cessation of azole therapy (Anderson, 2005). *In vitro* studies have also demonstrated a role for Hsp90, a molecular chaperone for calcineurin, in promoting the rapid acquisition of fluconazole resistance by *C. albicans* (Cowen & Lindquist, 2005; Cowen *et al.*, 2006).

Antifungal drug resistance is a stress response

The exposure of *C. albicans* to antifungal drugs will induce stress responses that are dependent on the dose and the nature of the drug. For fungicidal drugs such as echinocandins and polyenes at concentrations above the minimum growth-inhibitory concentration (MIC), the responses fail to prevent cell death. With fungistatic drugs, such as the azoles, at the MIC growth is inhibited but the cells are not killed – a phenomenon referred to in this context as drug tolerance (Sanglard *et al.*, 2003).

Most of the established antifungal resistance mechanisms are due to genetic mutation – usually point mutations in genes encoding drug targets or enzymes in metabolic pathways, or transcription factors leading to gene over-expression. Such mutations are stable, take time to be acquired, and can be thought of as long-term stress responses. Likewise, azole resistance can be caused by genetic rearrangements (Perepnikhatka *et al.*, 1999) or aneuploidy (Selmecki *et al.*, 2006) affecting the expression of drug targets, pumps or transcription factors. Antifungal drugs also, however, stimulate classic immediate stress responses. These can be thought of as reversible phenotypic responses that do not involve mutation or chromosomal rearrangement. Phenotypic switching and biofilm formation are other phenotypic responses that can increase drug resistance (Akins, 2005). The short-term phenotypic stress responses that lead to drug tolerance are important because they may allow *C. albicans* cells to develop long-term stable resistance mechanisms that could also confer a fitness gain.

An insight into the immediate stress responses induced by antifungals can be gained by transcript profiling cells exposed to drugs at, or below, their MIC. Liu *et al.* (2005) examined the effect of exposing *C. albicans* to 5-FC, amphotericin B, ketoconazole or caspofungin at concentrations equating to their IC_{50}s (concentrations that inhibit growth by 50 %). Exposure of cells to 5-FC resulted in increased expression of genes involved in purine and pyrimidine biosynthesis, including the gene implicated in resistance, *FUR1*, and the drug target *CDC21* (Liu *et al.*, 2005). Ketoconazole induced expression of genes involved in ergosterol biosynthesis, including *ERG3* and the target *ERG11*, the ABC drug efflux pumps *CDR1* and *CDR2*, and a putative sphingolipid flippase *RTA3*. The response of cells to amphotericin B was upregulation of genes involved in Ca^{2+}, K^{+}, Na^{+}, Zn^{2+} and sulphate transport and downregulation of phosphate, H^{+}, citrate, Cu^{2+} and Fe^{2+} transporters. In addition, there was upregulation of oxidative stress-response genes such as *AOX1, SOD2* and *GSH1*, consistent with amphotericin B causing oxidative damage (Sanglard & Bille, 2002). Caspofungin exposure induced the expression of several genes involved in cell wall maintenance, including the gene encoding the (1,3)-β-glucan synthase subunit *GSL1* (*GSL22*).

While it is not surprising that exposure to antifungals induced upregulation of drug targets and genes already identified as contributing to resistance, what is noteworthy is the number of other genes that were differentially expressed: 439 for 5-FC; 256 for amphotericin B; 82 for ketoconazole; and 480 for caspofungin exposure (Liu *et al.*, 2005). This in itself is also not surprising as the stresses will activate response networks involving transcriptional regulators with multiple substrates, as described above. It does raise the prospect, however, of identifying other potential drug targets including components of the stress-response pathways.

Antifungal drug tolerance

As already noted, the azoles are fungistatic and can be tolerated to a certain degree. If *C. albicans* calcineurin activity is inhibited, however, the azoles become fungicidal. The immunosuppressants cyclosporin A (CsA) and FK506 have been found to act synergistically with fluconazole (Sanglard *et al.*, 2003) and this is because they bind with either cyclophilin A (Cyp1p) or Rbp1p, respectively, and inhibit calcineurin by binding at the subunit A and B interface (Fig. 3), possibly restricting access of substrate to the active site (Cruz *et al.*, 2002; Sanglard *et al.*, 2003). Synergism of FK506 with fluconazole was also seen with *Candida tropicalis* and *Candida parapsilosis* but not with *S. cerevisiae* or *Candida krusei* (Cruz *et al.*, 2002). Calcineurin

is activated, following an increase in intracellular Ca^{2+}, by the binding of Ca^{2+}-calmodulin. As in *S. cerevisiae*, one of the major substrates of *C. albicans* calcineurin is transcription factor Crz1p (Karababa *et al.*, 2006). Crz1p is required for both calcium-induced transcription of *GSC1*, which may play a role in the repair of cell wall damage, and increased transcription of the gene encoding vacuolar Ca^{2+} P-ATPase pump Pmc1p (Sanglard *et al.*, 2003). This may provide feedback control of calcineurin activity (Figs 2 and 3). Disruption of *CRZ1*, however, did not completely remove azole tolerance, suggesting that the tolerance is mediated by other substrates of calcineurin (Karababa *et al.*, 2006; Onyewu *et al.*, 2004). Protein kinase Cka2p is involved in fluconazole susceptibility, possibly by inhibiting *CDR1* and *CDR2* expression (Bruno & Mitchell, 2005). In a *CKA2* knockout strain *CDR1, CDR2* and *RTA3* were overexpressed. Although calcineurin contributes to Cka2p-mediated fluconazole sensitivity, Crz1p is not the Cka2p substrate (Fig. 3). Thus the precise mechanism of calcineurin-dependent azole tolerance remains to be discovered. Calcineurin responds to changes in calcium ion concentration (Figs 2 and 3) and it is possible that exposure to azoles causes an intracellular calcium spike that induces the calcineurin tolerance pathway (Sanglard *et al.*, 2003).

Prospects of overcoming drug resistance

Clinically significant *C. albicans* drug resistance has only been observed to date with immunocompromised individuals prescribed long courses of azole antifungals. For the mechanisms resulting in high-level clinical resistance, described above, two strategies for overcoming the resistance are evident – inhibition of drug efflux pumps and other, indirect, ways of chemosensitizing cells to the azoles. Drug efflux pumps have been studied by heterologous expression in *S. cerevisiae*, where they confer increased resistance to azoles (Lamping *et al.*, 2007; Nakamura *et al.*, 2001; Niimi *et al.*, 2005; Schuetzer-Muehlbauer *et al.*, 2003). This not only allows an analysis of pump function (Lamping *et al.*, 2007) but also provides a system to screen for pump inhibitors that chemosensitize the strains overexpressing pumps to fluconazole (Monk *et al.*, 2002; Niimi *et al.*, 2004; Schuetzer-Muehlbauer *et al.*, 2003). While FK506 has been demonstrated to be a pump inhibitor using this approach (Schuetzer-Muehlbauer *et al.*, 2003) its other effects in the cell, such as inhibiting calcineurin-mediated azole tolerance, may complicate interpretation of chemosensitization susceptibility tests. Also, to be an effective azole chemosensitizer, pump inhibitors must be specific for the pumps causing resistance and not adversely affect human transporters. Inhibition of calcineurin-mediated azole tolerance has been proposed as a novel therapeutic approach (Steinbach *et al.*, 2007). While FK506 and CsA chemosensitize *C. albicans* cells to azoles, and can render the azoles fungicidal, they are also immunosuppressive, which could be problematic for candidosis patients, many of whom are already immunocompromised. Therefore the search needs to be widened to related chemosensitizers that are not immunosuppressants. The calcineurin stress-response pathway is extensive and provides other potential targets, such as Hsp90, although again inhibitors would have to be specific for *C. albicans* Hsp90.

Conclusions

Exposure of *C. albicans* to antifungal drugs induces immediate phenotypic stress responses that may permit drug tolerance. This tolerance allows the selection, or evolution, of stable stress responses that confer higher levels of resistance. All micro-organisms contain a plethora of genes that can potentially confer resistance to new environmental stresses (Wright, 2007). *C. albicans* is a diploid fungus with several gene families that have probably arisen by gene duplication. We have discovered that alleles of *CDR1* and *CDR2* within the same strain differ functionally and that *CDR2* alleles are continuing to evolve (Holmes *et al.*, 2006). Thus there are a large number of pumps with different substrate specificities that, if overexpressed or mutated, could confer resistance to 'novel' antifungals with intracellular targets.

This perspective on antifungal drug resistance suggests several possible ways of overcoming, or preventing, drug resistance. Combination therapy can be used to attack two different targets simultaneously with a low probability that resistance to both drugs will arise, or to attack a drug target and its resistance mechanism (cf. augmentin). While combination therapy sounds attractive, and it has been reported that antimicrobial combinations can actually select against the development of resistance (Chait *et al.*, 2007), clinical results are variable and there are increased efficacy testing requirements.

Another prospect for resistance prevention is to abrogate the initial stress response that permits tolerance and the development of resistance. Calcineurin, Hsp90 and other stress-response components provide a range of targets. A suitable tolerance inhibitor may render the azole fluconazole more potent and fungicidal, thus creating a wider window of therapeutic efficacy. Other potential targets include biofilm formation or transcriptional regulators of efflux pumps such as Tac1p, Upc2p or Mcm1p (Coste *et al.*, 2006; Riggle & Kumamoto, 2006; Silver *et al.*, 2004). Provided host toxicity is not a problem, selection of resistance can also be precluded clinically by simply prescribing high doses of antifungals.

A third strategy is to choose drug targets and select drug candidates more carefully. Rapidly acting fungicidal drugs are better, theoretically, than fungistatic drugs as they do not allow the selection of resistant variants. Therefore essential gene products make better targets than non-essential gene products. Extracellular targets are also better than intracellular targets as the intracellular action of drugs provides more options for drug inactivation, sequestration and efflux-pump-mediated resistance. Drug candidates should be screened as potential substrates of known drug

pumps (Niimi *et al.*, 2006), and the *in vitro* mutagenesis of the target, or selection of resistant host cells, could be used to give an indication of the likelihood of resistance developing (Anderson, 2005).

In summary, in order to ensure that novel antifungals are not merely additional stresses to which *C. albicans* will respond with tolerance and resistance, it is imperative to elucidate the biology, physiology and underlying stress responses of this important fungus.

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