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Drug resistance mechanisms and their regulation in non-*albicans Candida* species

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Fungal pathogens use various mechanisms to survive exposure to drugs. Prolonged treatment very often leads to the stepwise acquisition of resistance. The limited number of antifungal therapeutics and their mostly fungistatic rather than fungicidal character facilitates selection of resistant strains. These are able to cope with cytotoxic molecules by acquisition of appropriate mutations, re-wiring gene expression and metabolic adjustments. Recent evidence points to the paramount importance of the permeability barrier and cell wall integrity in the process of adaptation to high drug concentrations. Molecular details of basal and acquired drug resistance are best characterized in the most frequent human fungal pathogen, *Candida albicans*. Effector genes directly related to the acquisition of elevated tolerance of this species to azole and echinocandin drugs are well described. The emergence of high-level drug resistance against intrinsically lower susceptibility to azoles in yeast species other than *C. albicans* is, however, of particular concern. This is due to their steadily increasing contribution to high mortality rates associated with disseminated infections. Recent findings concerning underlying mechanisms associated with elevated drug resistance suggest a link to cell wall and plasma membrane metabolism in non-*albicans Candida* species.

Introduction

The rising incidence of multidrug resistance is a global threat to the effective treatment of human and animal infectious diseases of bacterial and fungal origin. High mortality associated with disseminated fungal infections is of particular concern, given the limited treatment options and the high adaptive capability of fungal pathogens to stress conditions associated with colonization of different host niches and drug exposure.

Longitudinal surveillance studies from various medical centres worldwide document the growing clinical relevance of yeasts of the genus *Candida* as the leading cause of fungaemia. *Candida albicans* remains the predominant causative agent of all forms of candidiasis. Epidemiological data, however, indicate the growing role of non-*albicans Candida* (NAC) as causative agents of nosocomial invasive candidaemias, altogether surpassing *C. albicans*.[1–4] Most infections attributed to NAC are caused by *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*. This changing epidemiology and shift towards species characterized by elevated, as compared with *C. albicans*, MICs of azoles, reflects their widespread use and prolonged prophylaxis in the growing population of high-risk patients.[5–7] The evolved virulence factors characteristic of fungal pathogens, combined with their great potential to develop antifungal resistance, account for the observed epidemiology. The incidences vary according to geographical region and patient group, but continue to increase worldwide.[8] High mortality (up to 60%), along with increasing resistance to antifungals among NAC species, poses serious economic and medical problems. This is related to the increase in the population of immunocompromised individuals, such as transplant-receiving patients, HIV-positive people and patients undergoing chemotherapy. Additional risk factors include prolonged treatment with broad-spectrum antibiotics, advanced age and premature birth. Frequent invasive medical treatments, use of implanted devices (valves, joints, catheters) and misuse of antifungal drugs also account for the observed shift towards isolation of NAC. Patient characteristics predisposing to infections caused by NAC are summarized in Table 1.

The opportunistic human pathogen *C. glabrata* is a primary species isolated from patients with a compromised immune system and the second most common aetiological factor for *Candida* infections.[20–23] Haploid and asexual *C. glabrata* live as commensals on mucosal surfaces, where they are a constituent of the normal microbiome.[24,25]

However, under suitable conditions *C. glabrata* may turn into an opportunistic pathogen causing superficial as well as deep-seated mycoses. It is most prevalent in transplant-receiving patients, individuals with non-transplant surgery and patients with solid tumours or haematological malignancies (Table 1). *C. glabrata* affects mostly older patients, diabetics or individuals pre-exposed to azoles or echinocandins, and is rarely isolated from neonates and young children. Mortality rates are higher in *C. glabrata* than in *C. albicans* infections, reaching 50% (Table 1). This is related to the enormous adaptability of *C. glabrata*. It may colonize many different host niches. In order to survive and proliferate it

Table 1. Infections caused by NAC species

Feature	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*
Average mortality rate	50% (30%–80%)	29%	40% (30%–70%)
Patient age	adults (>65 years)	premature infants, children and young adults (1–19 years)	adults (>60 years)
Underlying conditions	solid organ transplant, solid tumours, diabetes mellitus, haematological malignancies, pre-exposure to azoles or echinocandins, corticoid use	neutropenia, pre-exposure to echinocandins, parenteral nutrition, immunosuppressive therapy, burns, vascular catheterization, prosthetic devices, prior antibiotic therapy, prior surgery	neutropenia, organ transplant, haematological malignancies, prolonged catheterization
Major sites of infections	vagina, oral cavity, urinary tract, disseminated	gastrointestinal tract, oral cavity, disseminated	skin, oral cavity (AIDS and cancer patients), genitourinary tract, gastrointestinal tract, disseminated

Compiled using information from references 9–19.

Table 2. General features of NAC species

Feature	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*
Ploidy	haploid	diploid	diploid
Virulence factors			
adhesins	EPA family	ALS-like family	ALS-like family
hyphae/pseudohyphae	absent[a]	pseudohyphae	present
ability to form biofilms/form	moderate/mats	high/less structured, monolayer or multilayer	high/compact monolayer
major biofilm regulators	Bcr1	Bcr1, Cph2	Bcr1
secreted proteases	absent	SAPP1-3	SAPT1-4
drug resistance	natural azole resistance, acquired echinocandin resistance	increased echinocandin resistance, acquired echinocandin resistance	natural 5-fluorocytosine resistance, acquired fluconazole resistance, acquired echinocandin resistance

Compiled using information from references 34–42.
EPA, epithelial adhesins; ALS, agglutinin-like sequence; Bcr1, biofilm and cell wall regulator 1; SAP, secreted aspartyl proteases.
[a]Can form pseudohyphae under nitrogen starvation conditions.[34]

uses numerous mechanisms, allowing its successful adaptation as a pathogen. These include the ability to sustain long-term carbon and iron starvation upon phagocytosis by macrophages, as well as a low-pH environment in the vagina or phagolysosomes, increased resistance to nitrosative and oxidative stresses. On the other hand, relatively hypoxic conditions are encountered by this pathogen in some of the host niches it occupies, such as the periodontal space or intestine.[26,27] Finally, stress is posed by challenge with antifungal drugs used in medical treatment, as well as by competitive interactions with other microorganisms. These conditions are likely to affect the intrinsically low susceptibility of *C. glabrata* to fluconazole, which may quickly develop into high-level resistance during the course of medical treatment. Therefore, infections caused by *C. glabrata* are difficult to eradicate, leading to significant mortality combined with increased virulence.

One of the most prevalent aetiological factors causing nosocomial candidaemia in tropical countries is *C. tropicalis*.[9] This diploid, dimorphic fungal pathogen affects mostly neutropenic patients and individuals with haematological malignancies.[15,28] Candidaemia and invasive candidiasis caused by *C. tropicalis* are treated with amphotericin B or echinocandins. Although extended-spectrum triazoles are also in use, resistance is usually induced upon prolonged exposure.

C. parapsilosis, a diploid dimorphic yeast, is a normal human commensal; in contrast to *C. albicans*, *C. glabrata* and *C. tropicalis*, however, it can also live freely in a wide range of environmental niches. Recent data highlight the increasing clinical impact of this species. Multiple nosocomial outbreaks due to *C. parapsilosis* in clinical settings have been observed.[10,29,30] *C. parapsilosis* can disseminate via horizontal transmission. Exogenous sources such as medical devices and parenteral nutrition, but mostly the hands of healthcare workers, may be sources of fungaemia outbreaks.[31–33] *C. parapsilosis* affects individuals with a weakened immune system (Table 1); however, the group of patients at greatest risk of nosocomial infection with *C. parapsilosis* is that of low-birth-weight neonates.[11] A characteristic feature of this fungal pathogen is its high capability for biofilm formation on abiotic plastic surfaces (Table 2). This facilitates and enhances the

capacity of the organism to colonize prosthetic materials and indwelling devices, including catheters.[18,43] Difficult-to-eradicate *C. parapsilosis* biofilms, resistant to antifungal drugs, pose the need for removal of intravenous catheters and implanted devices.[44]

Molecular mechanism of drug resistance

The number of drug classes that can be used for the treatment of invasive fungal diseases is relatively small compared with the large number of antibacterial medications. Similar biology of the eukaryotic host and fungal pathogens leads to significant toxicity of certain drugs, frequently precluding their safe use over prolonged time, which is often required. Overuse of antifungals, which are most often applied as monotherapy, leads to selection of subpopulations of resistant cells, a process facilitated in patients with a weakened immune system.

Inherent resistance of certain species to antifungal drugs occurs naturally without pre-exposure to drug, whereas susceptible species can acquire resistance during medical treatment, which often leads to therapy failure. This acquired resistance to a single drug may be accompanied by cross-resistance to the entire group of antifungals of the same class, as well as to representatives of multiple structurally unrelated groups. Resistance to multiple drugs may also emerge in response to therapy approaches in which two or more drugs with different mechanism of action are applied sequentially or in combination. Resistance to both azoles and echinocandins in *C. glabrata* and *C. tropicalis* or azoles and amphotericin B in *C. glabrata* has been detected in clinical settings.[45–49] The emergence of MDR *Candida* (mostly among *C. glabrata* isolates) not responsive to treatment with all three classes of antifungal drugs is alarming.[50]

In order to survive the presence of toxic drugs, fungal cells have evolved various mechanisms, including target alteration, reduced uptake and active extrusion.[51] These mechanisms are connected with processes allowing protection of cell integrity and are coordinated with stress response signalling pathways. Stress adaptation promotes the evolution and maintenance of clinical drug resistance.

Resistance can develop to any of the three main classes of antifungal drugs that are in clinical use against infections caused by NAC species, namely echinocandins, azoles and polyenes.

Echinocandins, their mode of action and mechanisms of resistance

The primary and specific antifungal drug target is the cell wall. Its structure and composition are tightly regulated to reflect its multiple functions in pathogenic fungi. Enzymes engaged in the biosynthesis of cell wall components rapidly respond to any change in cell wall structure. This is effected by the coordinated action of several signalling pathways that sense and respond to defects within the cell wall, including those caused by some antifungal drugs.

Echinocandins disrupt cell wall biogenesis. This class of fungicidal drugs comprises micafungin, anidulafungin and caspofungin, the most widely used echinocandin.[52–54] These lipopeptides kill fungal pathogens via inhibition of the biosynthesis of an essential component of the fungal cell wall, β(1,3)-glucan.[55] The subunits of the integral plasma membrane protein 1,3-glucan synthase Fks1p or Fks2p are the target of specific non-competitive inhibition by echinocandins.[55] As a result, the fungal cell wall undergoes remodelling.

According to recent guidelines, echinocandins have been approved as the front-line clinical agents for candidaemia treatment.[56] This is due to their wide spectrum of fungicidal activity against most *Candida* species, including fluconazole-resistant strains. Although echinocandins have been in medical use since 2001 and resistance to this class of drugs appears rarely, an increasing number of NAC isolates exhibiting elevated tolerance has been reported.[57,58] Experimental data collected so far indicate that acquired echinocandin resistance in clinical isolates of *C. albicans*, *C. tropicalis* and *Candida krusei* is associated with spontaneous point mutations in the hot spot regions of the *FKS1* target gene.[59–62] Resulting amino acid changes reduce V_{max} of the glucan synthase enzyme.[59,62]

Strains of *C. parapsilosis* show reduced susceptibility to echinocandins due to the presence of a proline-to-alanine substitution at amino acid position 660 (P660A) within the HS1 region of Fks1p.[59] Therefore, further reduction of echinocandin susceptibility in clinical isolates of *C. parapsilosis* might involve regions of *FKS1* other than HS1 and HS2. Thus far, *FKS2* gene alterations have not been detected to play a role in echinocandin resistance in this yeast species.[63]

In clinical isolates of *C. glabrata*, and in contrast to other *Candida* species, echinocandin resistance involves mutations in the functionally redundant genes *CgFKS1* and *CgFKS2*.[64–66] The presence of clinically significant distinct mutations, mainly in the HS1 region of the *FKS*1 and *FKS2* genes, correlated with increased MIC of the echinocandin used and poor treatment outcome.[45,67]

Decreased echinocandin susceptibility can also result from mechanisms that are independent of *FKS* mutations. A phenomenon of conditional growth at high drug concentrations exceeding the MIC, with retention of full susceptibility at low to intermediate concentrations, named paradoxical growth or the Eagle effect, has been observed in *C. tropicalis*, *C. parapsilosis*, *C. albicans*, *Candida dubliniensis* and *C. krusei*. This has been linked to the induction of transient compensatory pathways leading to the accumulation of chitin and reduction of β(1,3)-glucan content in cells challenged with a high drug concentration.[66,68–70] Paradoxical growth depends on the type of echinocandin drug used and the targeted species.[68] Apart from altered cell wall structure, changes in morphology and growth rate have been attributed to the Eagle effect. Paradoxical growth is inhibited by the presence of human serum, and its *in vivo* significance remains uncertain.[71]

Yet another way of modulating echinocandin susceptibility involves membrane sphingolipids. While a high dose of echinocandins led to paradoxical growth, selection with low concentrations of caspofungin resulted in isolation of *C. glabrata* mutants exhibiting a mixed phenotype with reduced susceptibility to caspofungin (CRS) and increased susceptibility to micafungin (MIS). Genetically, the mixed phenotype was independent of the *FKS* genes and mapped to the loss-of-function mutations in genes encoding enzymes involved in sphingolipid biosynthesis (*FEN1*, *SUR4*, *SUR2* and *IFA38*). Accumulation of long-chain bases (LCBs), such as sphingosine and dihydrosphingosine, in CRS/MIS mutants was observed. Increased LCB content is likely to differentially influence interaction of caspofungin and micafungin with Fks hot spot regions embedded in the plasma membrane.

A role of LCBs in moderating echinocandin susceptibility has been shown in multiple yeast species, including *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. albicans* as well as moulds such as *Aspergillus nidulans*.[72–74]

Studies of the evolution of *C. glabrata* drug resistance to echinocandins in the human host revealed stepwise acquisition of accompanying compensatory mutations underlying increased resistance.[75] Global analysis of genome changes was performed on a series of *C. glabrata* isolates recovered from a patient undergoing multiple rounds of caspofungin treatment. WGS revealed the presence of a mutation within the drug target, *CgFKS2*, and a number of non-synonymous changes in genes previously not connected with echinocandin resistance. Apart from mutations in *CgFKS2*, alterations in *CgDOT6*, *CgMRPL11* and *CgSUI2* accompanied an increase in echinocandin resistance.[75] *CgFKS2* alone can confer echinocandin resistance. Based on the function of *Saccharomyces cerevisiae* orthologues that are involved in telomeric gene silencing (*ScDOT6*) or mitochondria organization (*ScMRPL11*), these processes might be involved in *C. glabrata* adaptation to the host or compensation of the adverse effect of *CgFKS2* mutation. A mutation that reduced the fitness cost of echinocandin resistance was identified in *CDC55*.

Loss of heterozygosity in *CtFKS1* has been observed during the course of echinocandin-based antifungal therapy against *C. tropicalis*. Environmental stress posed by caspofungin treatment led to *in vivo* selection and stepwise progression from *CtFKS1* to homozygosity for the *Ctfks1* mutation.[76] Loss of heterozygosity has been documented as an important factor in drug resistance evolution in *C. albicans*. Acquisition of hyperactive mutations in both alleles of the *TAC1* regulator increased azole resistance due to the overexpression of its downstream target genes *CDR1* or *CDR2*, encoding multidrug efflux pumps of the ATP-binding cassette (ABC) superfamily.[77] Thus, loss of heterozygosity can be considered as one of the mechanisms leading to stable drug resistance.[78]

Azoles, their mode of action and mechanisms of resistance

Azoles, including ketoconazole, itraconazole and fluconazole, have been the mainstay of antifungal therapy and prophylaxis for many years. A key azole drug, fluconazole has been in clinical use since the early 1990s. Its low toxicity, high efficiency and oral availability made it the most commonly used triazole compound.[79] New representatives of this class, voriconazole and posaconazole, which have an extended activity spectrum, have been approved for systemic treatment.[56] The fungistatic nature of azoles, along with their massive overuse in agriculture and medicine, facilitates the selection of resistant isolates. The transfer of field-selected azole-resistant *Aspergillus* to clinics has already been observed.[80–82] The huge selective pressure of azoles used in crop protection, associated with their long half-life and broad specificity, however, has global effects on multiple fungal species thriving in the environment, including those posing a threat to human health.[83]

Azoles inhibit cytochrome P450 14α-lanosterol demethylase, encoded by the *ERG11* gene and involved in ergosterol biosynthesis. As a result, ergosterol in the plasma membrane is depleted and methylated sterols, such as the toxic 14-α-methyl-ergosta-8-ene-3,6-diol, accumulate. This has a profound effect on membrane packing and increases membrane fluidity, which triggers severe membrane stress and affecting the function of numerous membrane proteins.[84]

Mechanisms of azole resistance include active efflux, target alteration or amplification and impaired drug uptake. Overexpression of efflux pumps of either the ABC superfamily or the major facilitator superfamily (MFS) leads to increased extrusion of drugs from cells, decreasing their concentration below toxic level.[85–89] Whereas some transporters, like *C. albicans CaMDR1*, of the MFS, are specific for the closely structurally related fluconazole and voriconazole, the ABC representatives show extremely broad substrate specificity, details of which remain unresolved.[90–93]

The mechanism of drug target alteration or amplification is connected to the ergosterol biosynthesis pathway. It is based on increased expression of the primary azole target *ERG11* or point mutations within the *ERG11* gene, which can decrease the affinity of azoles for the target enzyme. In addition, loss of the sterol Δ5,6-desaturase function coded by *ERG3* has been associated with decreased drug susceptibility.[94,95]

Altered drug uptake may reduce the intracellular azole level and influence the overall drug susceptibility status of the fungal cell. The exact molecular mechanism of this type is not entirely clear. According to early reports, azoles passively diffuse across the plasma membrane. Thus, altered plasma membrane lipid composition and subsequent changes in the permeability barrier would affect the uptake of drugs.[96,97] Recent studies, however, propose pH- and ATP-independent facilitated diffusion as a mechanism of entry of certain azole drugs. This previously uncharacterized mechanism was observed in clinical isolates of *C. albicans*, *C. krusei* and *Cryptococcus neoformans* and recently in *Aspergillus fumigatus*.[98,99] The saturation kinetics of fluconazole accumulation suggested that azole import was associated with energy-independent facilitated diffusion. Uptake of azole drugs can be influenced by their structure, hydrophobicity and polarity. Various accumulation (uptake) systems may therefore exist for drugs depending on their characteristics. Thus, changes in the lipid environment and membrane fluidity have the potential to affect both passive drug diffusion through the membranes and also protein-mediated facilitated diffusion, as the activity of membrane proteins largely depends on the membrane environment.

Each of the mechanisms described may operate separately in different isolates; however, the development of high-level resistance is usually achieved by their combined effect. Numerous recent experimental reports unravel new connections coupling azole resistance to fundamental processes involved in the pathogen's cellular homeostasis and allowing adaptive evolution.

Azole resistance in NAC species

Different NAC species vary in their susceptibilities to azoles. *C. tropicalis* and *C. parapsilosis* are regarded as being susceptible. Primary resistance to azoles is rare in these species. *C. glabrata* shows dose-dependent susceptibility or resistance and it is no longer considered to be susceptible to azoles.

Widespread prophylactic use of fluconazole coincided with an increased incidence of infections due to less susceptible NAC species.[100] Fluconazole-resistant clinical isolates of *C. glabrata*, *C. parapsilosis* and *C. tropicalis* have been detected worldwide.[21,101–103]

The development of azole resistance is relatively quick and dynamic. In addition, exposure of a susceptible clinical isolate to a specific azole could induce stable cross-resistance to the entire group of azole class of antifungals in all NAC species discussed.[103–105]

Thus far, there is limited information on the molecular mechanism underlying elevated azole tolerance in *C. parapsilosis* and *C. tropicalis*. The only global analysis of cellular traits that are altered upon azole treatment in *C. parapsilosis* resembles the description of molecular changes found in *C. albicans*.[104,106,107] It also indicates differences in cellular responses to specific azole antifungals used for treatment. Similarly to *C. albicans*, selection of *C. parapsilosis* towards increased fluconazole or voriconazole resistance led to the elevated expression of the *CpMRR1* transcription factor and its target *CpMDR1* and likely other MFS family members. In addition, noticeable up-regulation of enzymes from the family of aldo-keto reductases (AKRs) was observed in *MDR1*-overproducing isolates of both species.[104,106,107] Homologous stress-regulated AKRs are widespread in other organisms, including mammals, where they catalyse important redox modifications of various carbonyl-containing compounds, being engaged in their metabolism and detoxification.[108] The role of drug-induced AKRs in *Candida* remains to be clarified.

In contrast, posaconazole challenge of the same *C. parapsilosis* isolate triggered modifications in the composition of the plasma membrane, compensating for alterations in its fluidity and permeability. Posaconazole-induced inhibition of the production of ergosterol was balanced by overexpression of the transcriptional regulators *UPC2* and *NDT80*, which govern expression of the *ERG* genes of the ergosterol biosynthesis pathway.[104] Additional changes included the induction of *CpCDR3*—the homologue of *C. albicans* ABC transporter *CDR3*, which was shown to be engaged in out-to-in translocation of 7-nitrobenz-2-oxa-1, 3-diazole-4-yl (NBD)-labelled phospholipid analogues within the plasma membrane and *CpPDR16* phosphatidyloinositol transfer protein.[109]

Recent findings of Berkow *et al.*[110] suggest that azole resistance in *C. parapsilosis* clinical isolates depends on the combined specific changes in the ergosterol biosynthesis pathway due to the CpErg11 alteration (Y132F), increased expression of *CpERG11*, *CpCDR1* and *CpMDR1* transporters and the contributions of *CpTAC1*- and *CpMRR1*-dependent targets. The latest include members of the largely uncharacterized drug:H+ antiporter family of the MFS (DHA transporters), which are highly represented in *C. parapsilosis*. DHA transporters that have been linked to antifungal drug resistance in other pathogenic fungi include *C. glabrata FLR1*, *TPO3* and *QDR2* and *C. albicans CaMDR1* or *CaNAG3*.[92,111] Roles in ion homeostasis, virulence, biofilm formation and architecture or survival in the host have been attributed to MFS transporters, apart from mediating resistance to selected xenobiotics.[112] Together with members of the ABC transporter superfamily, they likely take part in cellular homeostasis and the stress response and might also act as a remote signalling and sensing system.

In clinical isolates of *C. tropicalis*, acquired *in vivo* azole resistance was related to increased expression of *CtERG11* or its missense mutations.[113,114] While alterations in the ergosterol biosynthesis pathway predominate *in vivo*, experimentally induced fluconazole resistance involved up-regulation of multidrug efflux pumps of either the ABC type (*CtCDR1*) or the MFS family (*CtMDR1*).[113] The process *of in vitro*-induced fluconazole resistance was very fast and dynamic but led to loss of virulence in the murine model, indicating reduced fitness of these mutants.

C. tropicalis with depletion of ergosterol in the plasma membrane and cross-resistance to polyenes and azoles has been identified in patients suffering from recurrent candidaemia.[48,49] Different missense mutations in *CtERG11* and *CtERG3* rather than active efflux led to selection of *C. tropicalis* accumulating 14-methyl sterols. Thus, altered plasma membrane composition and resulting changes in the permeability barrier are the key players in the acquisition of stable multidrug resistance in *C. tropicalis*.

Azole resistance in *C. glabrata*

In contrast to *C. albicans* and other NAC species, inherent as well as acquired azole resistance in *C. glabrata* clinical isolates relies predominantly on increased active drug efflux. This is due to elevated expression of the multidrug ABC transporter-encoding genes *CgCDR1*, *CgCDR2* and *CgSNQ2*.[87,88,114–118] Massive overexpression of a single ABC transporter gene or multiple multidrug ABC transporter genes in clinical isolates of *C. glabrata* resulted from spontaneous gain-of-function (GOF) mutations in the transcription factor *CgPDR1*, a homologue of *S. cerevisiae PDR1* and *PDR3*.[116–119] Early independent transcriptomic analyses of *C. glabrata* clinical isolates indicated that the main mechanism of azole resistance involves the *PDR1* regulon. This was in line with the very low frequency of mutations in other azole targets, such as *CgERG11* and *CgERG3*.[88,118–121] The status of the *CgPDR1* locus affects azole susceptibility in clinical settings, as recently revealed by high-throughput sequencing technology.[122]

Activating mutants of *CgPDR1*, apart from contributing to azole resistance *in vitro* and *in vivo*, modulate interaction with host cells. GOF mutations in *CgPDR1* increase virulence compared with the WT alleles in the murine model of disseminated candidiasis and enhance adhesion to epithelial cell lines, which might facilitate initial colonization of the host.[123,124] In addition, *CgPDR1* GOF mutants may promote evasion of the immune system by reduced adherence to and uptake by macrophages.[124]

Susceptibility to azoles is also connected with respiratory status in *C. glabrata*. It decreases in petite mutants with dysfunctional mitochondria. Mitochondrial dysfunction due to partial or complete loss of mitochondrial DNA (mtDNA) can be induced *in vitro* by exposure to ethidium bromide or the presence of azoles.[125,126] These *in vitro*-selected *C. glabrata* mutants emerged with high frequency.[127,128] Azole-resistant petite mutants of *C. glabrata* were also isolated from patients undergoing fluconazole therapy, although at low frequency.[126] *C. glabrata* petite mutants selected *in vivo* exhibited higher resistance with no need for GOF mutations within *CgPDR1*. They were also more virulent in the murine model.[128] In another approach to the analysis of the virulence of petite mutants of *C. glabrata* selected on drug-containing plates, reduced virulence was observed.[129] The discrepancy likely results from different selection procedures and the commonly observed heterogeneous nature of petite yeast mutants, which show increased mutation frequency.[130] Transcriptomic analysis of petite mutants indicated substantial overlap between *C. glabrata* and *S. cerevisiae*. Similarly to *S. cerevisiae*, defects in mtDNA led to increased expression of *CgPDR1* and concomitant up-regulation of nuclear genes connected with small-molecule transport and multidrug efflux as well as membrane lipid homeostasis. These include *CgCDR1*, *CgSNQ2*, *CgYOR1* and *CgPDR15*, encoding ABC transporters,

along with *CgPDR1* regulator and genes involved in lipid biosynthesis: *CgPDR16*, *CgIPT1*, *CgLCB5* and *CgLAC1*. The elevated expression of *CgPDR1* in petite mutants correlated also with up-regulation of *CgRTA1* and *CgRSB1*.[121,123,131] Both encode 7-transmembrane domain-containing proteins, which are overproduced in azole-resistant *C. glabrata*, *C. albicans* and *S. cerevisiae*. Rta1p and Rsb1p were initially identified in *S. cerevisiae* to increase resistance to 7-aminocholesterol and phytosphingosine, respectively, by an unknown mechanism.[131–135] The predicted topology of CgRta1p (and CgRsb1p) most closely resembles that of a family of ubiquitous 7-transmembrane helix G-protein-coupled receptors (7-TM GPCRs), which transduce various signals across biological membranes and in humans are the major target of therapeutic intervention by small-molecule drugs. It remains an open question whether CgRta1p and CgRsb1p are involved in G-protein-mediated signal transduction phenomena, functioning under defined stress conditions.

Comparison of recent data on CgPdr1 genomic binding sites in *C. glabrata* petite mutants obtained *in vitro* with a set of genes up-regulated in a mitochondrial mutant isolated from a patient indicated a core set of genes from the *PDR1* regulon, common to both variably selected mutants.[128,136] In both cases, massive overexpression of *CgCDR1* along with *CgPDR1* was observed. *In vivo* selection of highly resistant *C. glabrata* petites is connected with acquisition of many other changes that evolved to increase resistance and virulence in the mammalian host. These alterations affect the organization of mitochondria and cellular respiration, as well as the biogenesis, organization and maintenance of the cell wall.

Genetic alterations and variation in gene copy number contribute to azole resistance in *C. glabrata* and constitute a source of its enormous genetic diversity. Early reports indicated a positive correlation between the increase in copy number of *CgERG11* with elevated azole tolerance.[137] Rapid alterations in genomic organization, including karyotype changes during the time of infection, have also been reported.[138,139] The underlying presence of a considerable number of megasatellites or repetitive minisatellite sequences serving as a source of inter- and intra-chromosomal rearrangements leads to enormous genome plasticity. This, together with the enhanced genome mutability of haploid *C. glabrata*, allows fast adaptation and successful colonization of the host.

The picture of the response to azole antifungals has become even more complex in the light of recent findings. Components of the cell wall integrity (CWI) pathway and sterol biosynthesis and constituents of the RNA polymerase II mediator complex CgMed2p and CgGal11p were shown to be required for basal and acquired azole resistance in *C. glabrata*.[140] The RNA polymerase II mediator complex plays a crucial role in the process of transcription activation, where it serves as a bridge between upstream gene-specific regulatory proteins and the core of RNA polymerase II.[141] Removal of *CgMED2* impaired *CgPDR1* activation. It was also required for fluconazole resistance elicited by GOF *CgPDR1*. Similarly to the effect exerted by *CgGAL11* (*CgMED15*) disruption, it affected fluconazole-induced *CgPDR1* expression and led to reduced transcript levels for *CgCDR* genes.[140]

Cells lacking *CgMED2* were also more susceptible to caspofungin and constitutively activated the Pkc1p-mediated CWI pathway, indicating the involvement of upstream signalling cascades in the PDR response.[140] In this way, subtle perturbations in the cell wall and plasma membrane caused by the presence of drugs might be sensed and transmitted to CgPdr1p. Detailed molecular mechanisms of this interplay are yet to be discovered.

Another component of the mediator complex, the CgGal11p KIX domain, was shown to directly interact with CgPdr1p in a xenobiotic-dependent manner.[142] Direct binding of ketoconazole to ScPdr1p has been proposed as a mechanism triggering activation of its regulatory network in *S. cerevisiae*, including the azole resistance-conferring multidrug ABC transporter ScPdr5p. Although this is in line with the observation that ScPdr1p homologues, members of the Gal4 family, are ligand-activated transcription factors, the dissociation constant of ketoconazole was relatively high (39 μM).[142] Induction of transcriptional activators of multidrug transporters upon binding of xenobiotic substrates to regulatory proteins has also been observed in bacteria. Crystallographic analysis of the bacterial BmrR transcriptional activator of the multidrug transporter Bmr revealed the presence of a large electronegative pocket capable of accommodating multiple hydrophobic xenobiotics possessing a net positive charge with high affinity.[143,144] The compensatory activation of genes encoding multidrug ABC transporters in response to genetic inactivation of homologues of overlapping specificity was associated with specific activation of ScPdr1p, but without addition of xenobiotics.[145] This indicates that Pdr1p activation may involve interactions with endogenous ligands that have not yet been identified.

Polyene drugs, their mode of action and mechanisms of resistance

Polyenes are natural fermentation products of *Streptomyces*. The most commonly used polyene drug, amphotericin B, is connected with severe toxic side effects, including dose-dependent renal toxicity when used in the conventional way. Therefore, new liposome formulations with decreased toxicity and improved therapeutic index have been developed.[146,147] In comparison with other drugs, amphotericin B is refractory to the development of resistance. Despite nearly 50 years of use in medical treatment, resistance to amphotericin B is rare. The fungicidal action of amphotericin B is more complex than previously thought. According to the newly proposed sterol sponge model, it primarily forms large extramembranous aggregates that extract ergosterol from phospholipid bilayers, thus depleting cells of ergosterol.[148] Resistance to amphotericin B is very uncommon among NACs. Sporadic cases of enhanced amphotericin B tolerance were reported.[149] Membranes of *C. tropicalis* isolates resistant to azoles and with reduced susceptibility to amphotericin B were low in ergosterol.[48] In *C. albicans*, depletion of ergosterol led to the accumulation of C-14-methylated sterols or lanosterol.[150] In addition, amphotericin B induced the intracellular accumulation of reactive oxygen species (ROS) in NAC species.[151] Cells of *C. tropicalis* resistant to amphotericin B exhibited altered mitochondrial activity and produced significantly less ROS. It is, however, not clear whether these effects directly result from ergosterol depletion, which might affect mitochondrial membrane composition and function, or other amphotericin B-induced intracellular events. Multiple effects exerted by amphotericin B were reflected by decreased fitness and reduced virulence of *C. tropicalis* resistant to amphotericin B.[152] This was due to constitutive activation of the diverse stress responses imposed by amphotericin B challenge and the extremely high fitness costs of acquired mutations. Apart from compensatory

mutations, alterations were identified in the *CtERG2*, *CtERG3*, *CtERG5*, *CtERG6* and *CtERG11* genes of the ergosterol biosynthesis pathway. Ergosterol depletion activated compensatory mechanisms in the Hsp90 chaperone and Hsp90-dependent stress response pathways, including those of Hog1, calcineurin and Pkc1p CWI signalling.[152] This is in line with recent large-scale phenotypic profiling of the *C. glabrata* knock-out collection, unravelling genes linking modulation of amphotericin B tolerance to cell wall and lipid homeostasis.[153] Due to the essential role of ergosterol, various cellular processes underlying the fungicidal action of amphotericin B are likely to be affected.

Strikingly, an amphotericin B resistance mechanism dependent on plasma membrane proteolipid 3 (Pmp3) was recently proposed by Bari and co-workers.[154] Pmp3 is a conserved hydrophobic membrane polypeptide involved in maintenance of membrane potential and ion homeostasis. It was shown to specifically antagonize the amphotericin B effect in *C. glabrata* and *S. cerevisiae*.[155] Together with Pmp3, modulation of amphotericin B resistance was dependent on functional components of the sphingolipid biosynthesis pathway, such as the fatty acid elongases Fen1 and Sur4, the regulatory Ypk1 kinase and Sac1 phosphatidylinositol phosphate phosphatase.[156,157] Defects in *FEN1* and *SUR4* affect the entire yeast lipidome, leading to accumulation of aberrant sphingolipid species and compromised vacuolar and protein trafficking to the cell surface.[158] The concomitant adaptational modifications of ergosterol and sphingolipid metabolism are likely due to their critical involvement in raft formation.

Drug resistance and cell wall stress signalling

As key enzymes involved in cell wall biogenesis are associated with the plasma membrane, and the two structures remain in close contact during cell growth, providing a physical barrier between the cell interior and the outside world, the homeostasis of the two structures requires coordinate regulation. Integrity of the cell wall, providing mechanical rigidity, is crucial for cell survival and it must therefore be appropriately remodelled in response to adverse external stresses, including that posed by antifungal drugs. Alterations in the cell wall are sensed by plasma membrane-bound sensors that trigger activation of the signalling cascades (Figure 1).

General signalling pathways, like the protein kinase C (PKC1)/CWI/mitogen-activated protein (MAP) kinase cascade, the HOG signalling and calcium/calmodulin-dependent phosphatase calcineurin pathways, as well as the TOR pathway, are highly conserved among different yeasts, including NAC species. Signalling pathways are interconnected, coordinately regulating their target genes involved in crucial cellular functions, including those associated with membrane and cell wall metabolism.

The primary function in modulating the response to echinocandins and azoles is played by the PKC1 cascade, which is known to control CWI. Defects within the cell wall or transient compensatory changes induced by high caspofungin concentration activate PKC/CWI along with calcineurin pathways in *C. albicans*, *S. cerevisiae*, *C. krusei*, *C. parapsilosis* and *Candida guilliermondii*.[159,160] Similarly, in *C. glabrata*, rescue from fungicidal concentrations of echinocandins was associated with activation of the CgSlt2p MAPK, in the Pkc1p kinase cascade, along with its target, CgRlm1p transcription factor.[68,161–163] Another downstream target of CgSlt2p, CgSBF (Swi4–Swi6 cell cycle box binding factor), seemed to contribute to micafungin resistance.[164] More experiments, however, are needed to clarify the exact role of CgSBF components in resistance to echinocandins as well as their interplay with CgRlm1p.

The role of the PKC/MAPK cascade in azole susceptibility was shown in *C. albicans*, *S. cerevisiae* and *Cryptococcus neoformans*.[165–167] Among NAC species, a direct link between CWI

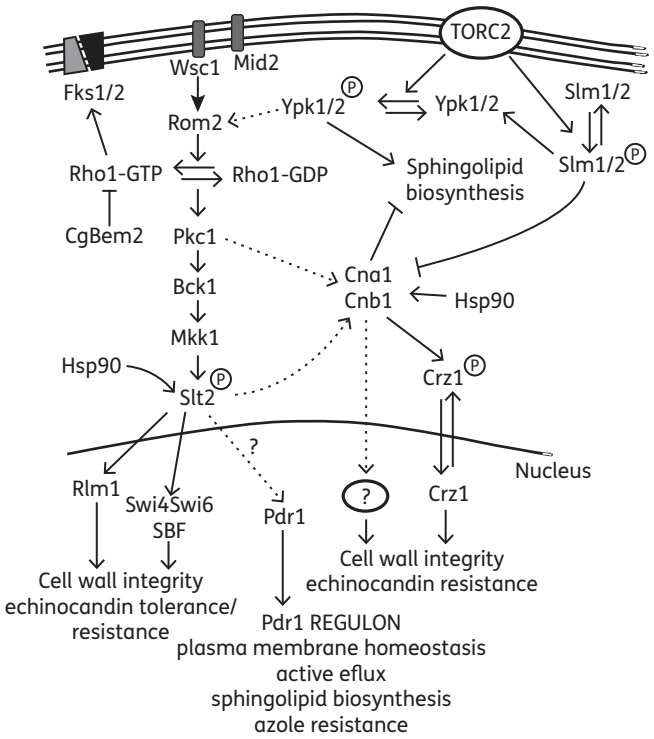

Figure 1. Schematic diagram of Pkc1p, calcineurin and TORC2 signalling pathways involved in modulation of drug resistance. The PKC/CWI, calcineurin and TORC2 pathways control a number of cellular processes, including plasma membrane integrity and cell wall homeostasis. Membrane sensors (e.g. Wsc family, Mid2) detect alterations in the cell wall and trigger signalling pathways. The PKC pathway plays a primary role in the response to echinocandin and azole challenge. Rho1 GTPase activates the PKC cascade (MAPKK Bck1, MAPK Mkk1 and MAP kinase Slt2). Rho1p also acts as a regulatory subunit of Fks1p/2p glucan synthase. Activated MAP kinase Slt2p modulates the activity of transcription factors that control the expression of genes involved in cell wall biogenesis. In *C. glabrata*, Slt2p modulates azole tolerance via the CgBem2p regulatory protein. CgSlt2 might influence the activity of the CgPdr1 transcription factor, which controls genes from the PDRE regulon (dotted arrow). The phosphatase calcineurin, composed of two subunits, Cna1p and Cnb1p, is activated by calcium/calmodulin (not shown on the diagram). Calcineurin dephosphorylates the Crz1p transcription factor, which in turn moves into the nucleus and induces expression of genes involved in CWI. Calcineurin inhibits sphingolipid biosynthesis via dephosphorylation of Lag1p and Lac1p ceramide synthases. Slm1p/2p from the TORC2 signalling network bind to calcineurin and inhibit its activity. Downstream substrates of TORC2, i.e. Ypk1/2 kinases, control the Rho1p GTPase switch most probably via Rom2p (dotted arrow). Rho1p couples TORC2 signals to the MAPK module. Solid arrows, confirmed positive regulation; dotted arrows, suggested positive regulation; bar-headed arrows, confirmed negative regulation.

pathways and response to azole stress was demonstrated in *C. glabrata*.[168] Components of the PKC cascade like CgBem2p, which regulates GTPase CgRho1p or MAPK CgSlt2p, were shown to modulate azole susceptibility. Decreased tolerance to azole antifungals in *Cgbem2Δ* cells appeared to be connected with the inability to induce expression of ABC multidrug efflux pumps and their transcriptional regulator *CgPDR1*, thus linking the PDR regulon with PKC1 signalling. ScBem2p, along with proteins involved in MAPK signalling, was also detected in a screen directed towards identification of upstream modulators of the PDR network or compensatory pathways independent of Pdr1p/Pdr3p in *S. cerevisiae*.[169] However, detailed interplay between the PDR-mediated response to drugs and kinase pathways remains to be further investigated.

In addition to monitoring the integrity of the cell wall, the core components of the PKC/MAPK pathway, namely Rho1p, Pkc1p and Tus1p, participate in membrane fluidity homeostasis, which is needed for proper environmental adaptation.[170] Recently, proteomic analysis revealed Pkc1p-induced phosphorylation of eisosome components ScPil1p and ScLsp1p, thus showing a connection between Pkc1p signalling and furrow-like plasma membrane microdomains.[171] Other proteomic studies showed that fluconazole affects not only the cell membrane but also influences the composition of the fungal secretome and the cell wall.[172] Analysis of the *C. albicans* secretome and cell wall subproteome upon fluconazole challenge suggested morphotypic changes due to the decreased levels of proteins associated with hyphal formation (CaAls3p, CaHwp1p, CaPlb5p) in the cell wall and thus favoured yeast growth. Concomitantly, increased incorporation of glycosylphosphatidylinositol (GPI)-anchored aspartyl protease CaSap9p or proteins involved in cell wall repair (CaPhr1p, CaPhr2p, CaPga4p, CaPir1p) was observed. These data, together with the reduced level of glucan, indicate severe remodelling of the cell wall upon challenge with fluconazole.[173] It is likely that similar changes might take place in related NAC species.

Apart from the PKC cascade, serine/threonine phosphatase calcineurin modulates tolerance to echinocandins and azoles in many yeast species. Calcineurin and its main downstream effector, Crz1p transcription factor, play diverse roles in the response to drug challenge among different *Candida* species. In *C. tropicalis*, Crz1p has a dual function: it is needed for micafungin tolerance and is dispensable for responses to caspofungin, anidulafungin and azoles.[174] In contrast, *C. glabrata crz1Δ* mutants exhibited high-level azole resistance, suggesting its negative role in the response to these drugs.[153,175] Thus, subtle differences between species may indicate the involvement of other, yet unidentified, interactions between calcineurin and client proteins modulating drug-specific responses (Figure 1).

The catalytic subunit of calcineurin is stabilized by heat shock protein Hsp90p, a molecular chaperone known as a global regulator of cellular signalling and stress responses in eukaryotic cells.[175–177] The critical role of Hsp90p in basal and acquired azole tolerance was shown in *C. albicans* and *S. cerevisiae*.[178] Inhibition of calcineurin or depletion of Hsp90p function abolished azole resistance. In *C. glabrata* Hsp90p and calcineurin regulation is necessary for basal as well as acquired tolerance to echinocandins.[75,162] Loss of calcineurin function or pharmacological inhibition of Hsp90p reduced echinocandin resistance despite the presence of the *CgFKS2* mutant allele.[75]

The TOR complex 2 (TORC2)-activated Ypk1 signalling cascade is a common pathway involved in the response to induced plasma membrane stress and a link with the calcineurin and PKC pathways (Figure 1). Our knowledge of these interactions comes mainly from research on *S. cerevisiae*. A single study of Ypk1p function in NAC comes from the analysis of *C. glabrata* cells with deletion in *YPK1*, which were hypersensitive to azoles, echinocandins and amphotericin B, suggesting its general role in cellular responses to stress posed by antifungal drugs.[153] In *S. cerevisiae* Ypk1p regulates polarization of the actin cytoskeleton and endocytosis, which are also affected by sphingolipids.[179–181] The essential function of Ypk1p is the regulation of sphingolipid biosynthesis.[182] This occurs at several steps of the pathway, including the first committed reaction catalysed by the serine palmitoyl transferase SPT, a complex of Lcb1p, Lcb2p and Tsc3p. Ypk1 activates SPT by phosphorylation of its inhibitory proteins Orm1p and Orm2p, resulting in their dissociation. Ypk1p also phosphorylates ceramide synthases Lac1p and Lag1p, with preference for Lac1p.[183] Interestingly, some of the steps activated by Ypk1p are common to those activated at the level of transcription by Pdr1p, which include *LCB2* and *LAC1*.[133,184] *CgLAC1* as well as *CgLCB5*, which encodes sphingoid long-chain base kinase, are also Pdr1p targets in *C. glabrata*.[118]

In *S. cerevisiae*, activation of Ypk1p takes place in response to compromised sphingolipid biosynthesis and involves phosphorylation by the eisosome-associated protein kinase Pkh1p or interaction with Slm1p/Slm2p regulatory proteins.[185] Calcineurin negatively regulates sphingolipid production by direct dephosphorylation of Lac1p and Lag1p.

Ypk1p negatively regulates endocytosis via inhibition by phosphorylation of Fpk1/2 kinases. Fpk1 and Fpk2 are known as activators of aminophospholipid flippases Dnf1p and Dnf2p, involved in maintenance of phospholipid asymmetry. Interestingly, the function of these flippases was shown to be negatively regulated by Pdr5p and Yor1p MDR transporters, indicating that these ABC pumps play roles, additional to drug transport, affecting the function of other plasma membrane proteins.[186]

Conclusions

Epidemiological data clearly indicate the growing importance of NAC-caused infections worldwide. Given the increasing number of immunocompromised patients and increasing antifungal drug resistance, there is an urgent need for the development of new effective treatments. Better understanding of basic fungal biology and pharmacotherapy adaptation mechanisms, facilitated by progress in new technologies, including deep sequencing, has the potential to highlight the dynamic robust changes in fungal pathogens during the course of therapy. High-throughput postgenomic technologies in combination with reverse genetics and the development of classical molecular tools dedicated to each pathogenic NAC species should allow the detailed evaluation of fungal adaptation to the infected host, depending on the occupied niches and type of drug used, and speed up the process of development of new antifungal approaches.

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Transparency declarations

None to declare.

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