Molecular Microbiology (2003) **48**(1), 225–235

The *Candida albicans* Cdr2p ATP-binding cassette (ABC) transporter confers resistance to caspofungin

Manuela Schuetzer-Muehlbauer,[1] Birgit Willinger,[2] Gerd Krapf,[1] Sabine Enzinger,[1] Elisabeth Presterl[3] and Karl Kuchler[1]*

[1]*Institute of Medical Biochemistry, Department of Molecular Genetics, University and BioCenter of Vienna, Dr Bohr-Gasse 9/2, A-1030 Vienna, Austria.*
[2]*Division of Clinical Microbiology, Institute of Hygiene and Medical Microbiology, and* [3]*Department of Medicine I, Division of Infectious Diseases, University of Vienna, A-1090 Vienna, Austria.*

Summary

Multidrug resistance may pose a serious problem to antifungal therapy. The *Candida albicans* Cdr2p is one of two ATP-binding cassette (ABC) transporters mediating antifungal resistance *in vivo* through increased drug efflux. Echinocandins such as caspofungin represent the newest class of antifungals that target cell wall synthesis. We show here by agar plate resistance assays that cross-resistant clinical isolates of *C. albicans* display high minimal inhibitory concentrations (MICs) to caspofungin when compared with a sensitive ATCC reference strain. Northern analysis and immunoblotting indicate that these isolates also show high levels of *CDR1* and *CDR2* expression. To determine a possible contribution of Cdr1p or Cdr2p to caspofungin resistance, we have functionally expressed Cdr1p and Cdr2p in appropriate recipient strains of the yeast *Saccharomyces cerevisiae*. Yeast cells expressing Cdr1p or Cdr2p exhibit cross-resistance to established antifungal drugs such as azoles and terbinafine. However, Cdr2p and, to a much lesser extent, Cdr1p confer caspofungin hyperresistance when expressed in yeast. Likewise, Cdr2p confers caspofungin resistance when constitutively overexpressed in a drug-sensitive *C. albicans* strain. We therefore propose that Cdr2p may contribute to clinical candin resistance. Finally, our data suggest that cross-resistance phenotypes of clinical isolates are the consequence of distinct mechanisms that may operate simultaneously.

Accepted 10 December, 2002. *For correspondence. E-mail kaku@mol.univie.ac.at; Tel. (+43) 1 4277 61807; Fax (+43) 1 4277 9618.

Introduction

Opportunistic pathogenic *Candida* spp. can cause life-threatening infections in immunocompromised individuals. Although oropharyngeal infections are most common, systemic or invasive candidiasis is also often found (Bastert *et al*., 2001). *Candida albicans* appears as the most prevalent cause, although other species such as *Candida glabrata*, *Candida krusei* and *Candida tropicalis* occur with increasing frequency (Wingard *et al*., 1993). Antifungal azoles are mainly used to treat systemic mycoses because of their good safety profiles. They block ergosterol biosynthesis through inhibition of the Erg11p cytochrome P450 14α-lanosterole demethylase, thus leading to a depletion of plasma membrane ergosterol (Heimark *et al*., 2002). Further, flucytosine fights invasive mycoses (Chiou *et al*., 2000), whereas the allylamine terbinafine (Petranyi *et al*., 1984; Ryder, 1999) is mainly used for topical applications against dermatophytes (Ryder *et al*., 1998). Effective antimycotic therapy also uses amphotericin B (AmB; Ellis, 2002), which appears to interact with ergosterol, hence rendering cells leaky. Unfortunately, AmB has life-threatening side effects such as nephrotoxicity and is therefore only used as a last resort in cases where azole treatment fails (Louie *et al*., 2001).

The intensive clinical use of azoles in both therapy and prophylaxis favoured resistant strains and non-susceptible *Candida* spp. to emerge with increasing frequency (Sanglard and Odds, 2002). Moreover, the fungistatic action of azoles facilitates genetic adaptation, leading to clinical drug resistance that involves several mechanisms (White *et al*., 2002). Besides mutations in *ERG11* or altered drug uptake resulting from permeability changes, enhanced drug efflux seems to be a frequent resistance mechanism. Azole treatment induces two genes encoding ATP-binding cassette (ABC) transporters, *CDR1* and *CDR2*, as well as the major facilitator permease *BEN*^{R} (*MDR*) (Prasad *et al*., 1995; Sanglard *et al*., 1995; Sanglard *et al*., 1997), all of which are implicated in clinical azole resistance in *Candida* spp. (Sanglard *et al*., 1996). Cdr1p and Cdr2p, orthologues of the yeast pleiotropic drug resistance

transporters Pdr5p and Snq2p (Bauer *et al*., 1999), act as ATP-dependent pumps with azole efflux capacity (Sanglard *et al*., 1996). Moreover, Cdr1p/Cdr2p mediate cross-resistance to unrelated drugs including terbinafine when expressed in yeast (M. Schuetzer-Muehlbauer *et al*., unpublished data; Nakamura *et al*., 2001).

The threat of increasing antifungal resistance prompted the development of new drugs such as the echinocandins (Kurtz and Douglas, 1997), but also stimulated novel global strategies for drug target identification (De Backer *et al*., 2001). Candins inhibit cell wall synthesis through a block of the β-1,3 glucan synthase (Kurtz and Douglas, 1997), hence acting fungicidally (Bartizal *et al*., 1997). They exhibit a broad antifungal spectrum, being effective against most *Candida* spp., *Aspergillus* spp. (Abruzzo *et al*., 1997) and *Pneumocystis carnii* (Powles *et al*., 1998), whereas *Cryptococcus neoformans*, *Fusarium* spp. and *Trichosporon* spp. (Denning, 2002) are unaffected. Notably, genetic selection for candin tolerance *in vitro* led to the isolation of several echinocandin-resistant *Saccharomyces cerevisiae* (Douglas *et al*., 1994a) and *C. albicans* strains (Kurtz *et al*., 1996). The genetic alterations were always mutations in orthologous genes encoding the β-1,3 glucan synthase subunits, Fks1p or CaFks1p respectively. The membrane-bound catalytic subunit Fks1p, together with the soluble regulatory subunit Rho1p, form an active glucan synthase (Douglas *et al*., 1994b; 1997; Mio *et al*., 1997) in vegetatively growing cells, whereas Fks2p is necessary during sporulation and under starvation (Mazur *et al*., 1995).

Candida albicans clinical isolates often display cross-resistance to azoles and several other unrelated drugs (White *et al*., 2002). Candins are thought to bypass resistance problems, as they are active even when azoles or AmB fail (Louie *et al*., 2001). Clinical candin resistance, as well as terbinafine resistance, has indeed not been reported as yet. However, we demonstrate here that azole-resistant clinical isolates with high levels of Cdr1p and Cdr2p also display high minimum inhibitory concentrations (MICs) for caspofungin. Functional expression of both efflux pumps in *S. cerevisiae* reveals that Cdr2p mediates pronounced caspofungin resistance. This is the first report showing ABC transporter-mediated candin resistance, and the first evidence for distinct antifungal substrate preferences of the Cdr1p and Cdr2p ABC efflux pumps.

Results

Azole-resistant clinical isolates show high mRNA levels of CDR1 *and* CDR2

We have collected numerous clinical isolates of *Candida* spp., some of which display high MIC values for azoles such as fluconazole, ketoconazole or itraconazole. The clinical isolates were identified by biochemical and morphological methods, and three isolates of *Candida albicans*, displaying MICs to fluconazole of more than 32 mg l^{-1}, were subjected to further analysis (Table 1). The corresponding ketoconazole MICs were 1–2 mg l^{-1}, indicating cross-resistance to several azole antifungals. To determine whether these strains have elevated mRNA levels of the ABC transporter genes *CDR1* and *CDR2*, we performed Northern analysis. Total RNA was prepared from clinical isolates, as well as the reference strain ATCC44374, and equal amounts were subjected to Northern blotting (Fig. 1A). We used staining of ribosomal RNA to verify equal RNA loading (Fig. 1A). The *CDR1* mRNA was barely detectable in ATCC44374 (Fig. 1A), and *CDR2* was only visible after prolonged exposure of blots (data

Table 1. Quantification of relevant drug resistance phenotypes in fungal strains of this study.

	Minimal inhibitory concentrations (MIC) (mg l^{-1})			
	Fluconazole	Ketoconazole	Itraconazole	Caspofungin
NCCLS method M27-A				
C. albicans ATCC44374	0.5	0.03	0.12	0.48
C. albicans 'B'	128	2	1	0.48
C. albicans 'f'	32	1	0.5	0.48
C. albicans 'i'	32	1	1	0.48
Agar plate assays				
C. albicans ATCC44374	ND	ND	ND	≈ 0.1
C. albicans 'B'	ND	ND	ND	0.35
C. albicans 'f'	ND	ND	ND	0.4
C. albicans 'i'	ND	ND	ND	0.15
S. cerevisiae control	ND	ND	ND	≈ 0.1
S. cerevisiae Cdr1p	ND	ND	ND	0.45
S. cerevisiae Cdr1p-GFP	ND	ND	ND	≈ 0.1
S. cerevisiae Cdr2p	ND	ND	ND	1
S. cerevisiae Cdr2p-GFP	ND	ND	ND	0.6

MIC values (mg l^{-1}) are given for *C. albicans* and *S. cerevisiae* strains listed in the first column. Quantification was done according to the NCCLS protocol as described in *Experimental procedures* or by agar plate resistance assays (Figs 2 and 4). ND, not determined.

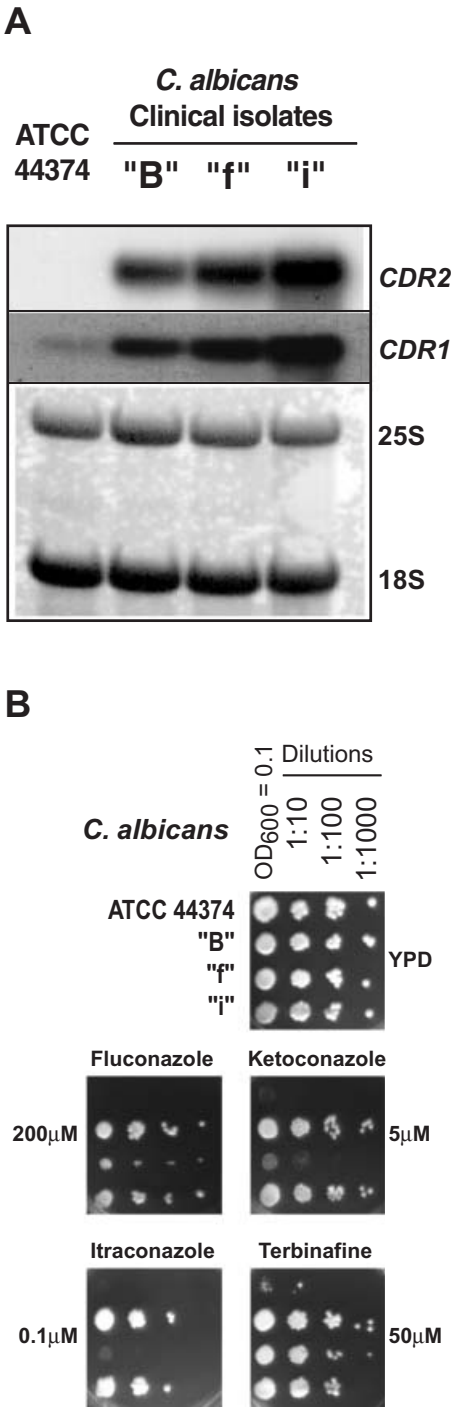

Fig. 1. *CDR1* and *CDR2* are upregulated in resistant clinical *C. albicans* isolates.
A. Northern analysis of total RNA. Cells were grown overnight in liquid Sabouraud and recultivated to the exponential growth phase after dilution to an OD_{600} of 0.1. Total RNA was prepared from 10 OD_{600} of cells, and 20 μg aliquots were subjected to Northern blotting using radiolabelled *CDR1* (nt 95–576) or *CDR2* (nt 10–468) probes.
B. Growth inhibition assays of azole-resistant *C. albicans* clinical isolates and the reference strain ATCC44374 after growing for 48 h at 30°C. Exponentially growing cells were diluted to OD_{600} = 0.1 and spotted in 10-fold serial dilutions onto Sabouraud plates containing antifungal drugs.

not shown). This is consistent with the low azole MIC of ATCC44374 (Table 1). In contrast, *CDR1/CDR2* mRNA levels, as well as corresponding protein levels (data not shown), were much higher in the clinical isolates than in the reference strain. Quantification by laser scanning densitometry of *CDR1/CDR2* mRNA levels relative to the reference strain indicated at least an order of magnitude higher levels in clinical isolates 'B', 'f' and 'i', with variations in each isolate, but 'i' showing the highest expression of both genes (data not shown).

We then used agar plate resistance assays to show that strains also displayed higher resistance to antifungals, including fluconazole, itraconazole and ketoconazole, as well as terbinafine (Fig. 1B). Although the ATCC44374 reference strain grew only on control plates lacking drugs (Fig. 1B), clinical isolates 'B' and 'i' displayed pronounced azole and terbinafine resistance. However, isolate 'f' was terbinafine resistant but remained azole sensitive, when compared with ATCC44374. We emphasize that the most appropriate reference strain, i.e. congenic sequential isolates from the same patients, were unavailable for technical reasons. Thus, any relative mRNA increases, resistance phenotypes and resulting changes in MIC values have to be interpreted with caution. Nevertheless, our results demonstrate that cross-resistant clinical isolates display reduced azole as well as terbinafine sensitivities *in vitro*.

The azole-resistant isolates show cross-resistance to caspofungin

We then tested whether cross-resistant clinical isolates also exhibit resistance to caspofungin, a new antifungal drug most recently approved for therapy. Thus, we checked the isolates 'B', 'f' and 'i' for their growth properties in the presence of caspofungin acetate on Sabouraud agar plates (Fig. 2). The caspofungin concentrations ranged from 0.1 to 0.5 μg ml^{-1} (80–400 nM). The clinical isolate 'i' was almost as sensitive to caspofungin as the reference strain, as it failed to grow at 0.3 μg ml^{-1}, corresponding to a MIC of about 0.1 μg ml^{-1}. In contrast, the isolates 'B' and 'f' grew at 0.3 μg ml^{-1} and even up to 0.5 μg ml^{-1} caspofungin (Fig. 2). Notably, isolate 'f' displayed the highest caspofungin MIC, but showed the lowest azole resistance (Figs 1B and 2). Yet, both 'f' and 'i' harbour high *CDR1*/*CDR2* mRNA (Fig. 1A), as well as protein levels (data not shown). These data indicate that overexpression of resistance genes such as *CDR1* and *CDR2* in clinical isolates does not always correlate with corresponding hyper-resistance or with the underlying efflux mechanism, most probably because of complex genetic backgrounds and history of selection in different patients *in vivo*.

Functional expression of Cdr1p and Cdr2p in yeast

To test a possible contribution of Cdr1p or Cdr2p to caspo-

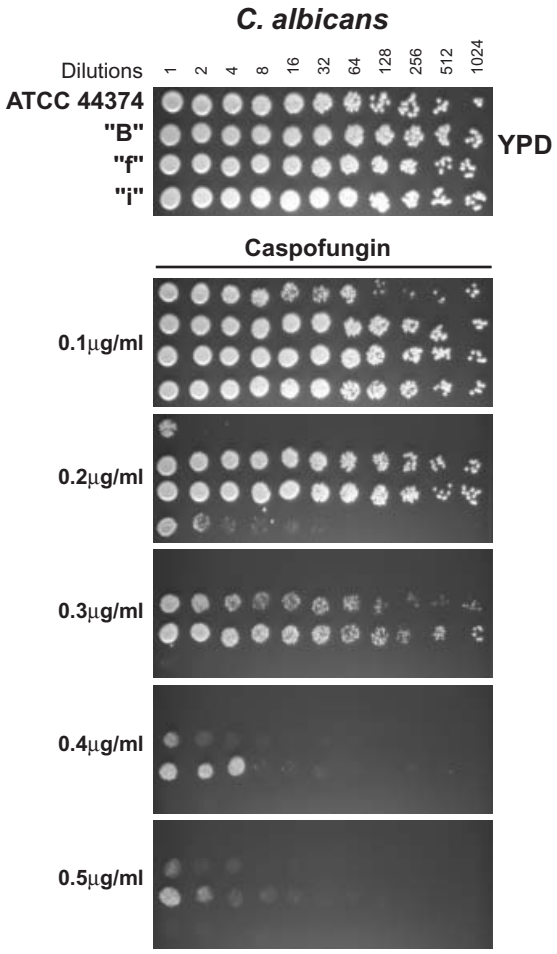

Fig. 2. *Candida albicans* clinical isolates are cross-resistant to caspofungin. Drug sensitivity tests were carried out with *C. albicans* clinical isolates grown overnight in liquid Sabouraud, followed by dilution to an OD_{600} of 0.1 and recultivation to the exponential growth phase. Aliquots (5 μl) of a 0.1 OD_{600} culture (≈ 5000 cells per spot) were spotted on drug-containing plates, along with twofold serial dilutions ranging from 1:2 to 1:1024. Colonies were inspected after growth for 48 h. The reference strain ATCC44374 was used as a wild-type control.

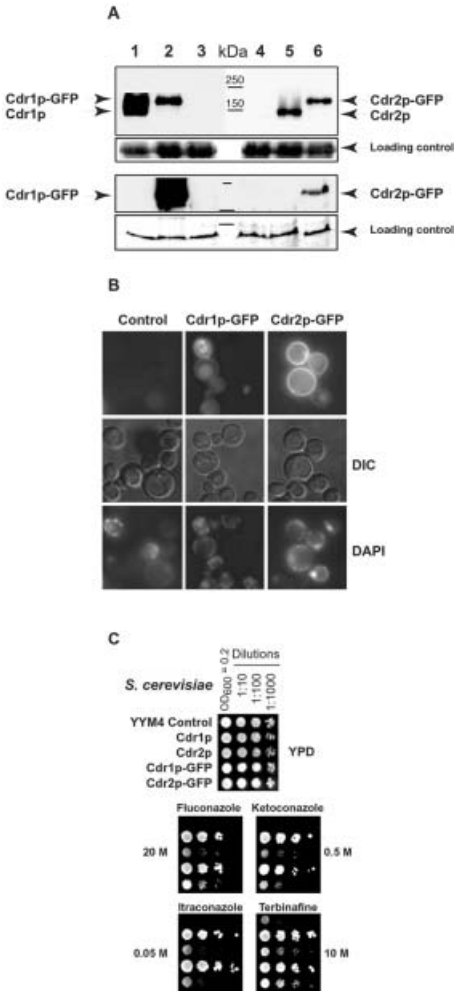

Fig. 3. The Cdr1p and Cdr2p ABC efflux pumps are functional in *S. cerevisiae*.
A. Western blot of extracts from *S. cerevisiae* cells (YYM4) expressing Cdr1p, Cdr2p or the GFP-tagged variants (Cdr1p-GFP, Cdr2p-GFP). Cell-free extracts corresponding to OD_{600} = 0.5 of cells were separated by SDS-PAGE in 7.5% gels, blotted to nitrocellulose membranes and detected with polyclonal antisera against Cdr1p or Cdr2p respectively. In addition, yeast extracts corresponding to 2 OD_{600} were also blotted and detected with a monoclonal anti-GFP antibody to compare expression levels of Cdr1p-GFP and Cdr2p-GFP. Lane 1, Cdr1p; lane 2, Cdr1p-GFP; lanes 3 and 4, control (empty vector); lane 5, Cdr2p; lane 6, Cdr2p-GFP. Unspecific cross-reactions to the antibodies served as loading controls.
B. Fluorescence microscopy of GFP-tagged versions of Cdr1p and Cdr2p. Cdr1p-GFP and Cdr2p-GFP localize to the plasma membrane. Fluorescence microscopy pictures (FITC filter) were taken from yeast cells (YYM4) harbouring plasmids driving the expression of Cdr1p-GFP or Cdr2p-GFP or the empty vector control. Cells were grown on plates containing adenine to reduce background autofluorescence of the vacuole. Microscopy was performed in a Zeiss Axiovert 135 microscope equipped with a DIC, FITC and DAPI filter set. Nuclear DNA was stained with DAPI.
C. Growth inhibition assays of *S. cerevisiae* cells (YYM4) harbouring multicopy plasmids driving the expression of Cdr1p or Cdr2p or the empty vector control. Cells were grown in selective medium to the exponential growth phase. 0.2 OD_{600} cultures, as well as 10-fold serial dilutions, were spotted onto YPD plates containing the indicated drug concentrations. Colony growth was detected after a 72 h incubation at 30°C.

fungin resistance, we took advantage of the genetically defined model organism *S. cerevisiae* suitable for the functional expression of *CDR1* and *CDR2* (Sanglard *et al*., 1995; 1997; Nakamura *et al*., 2001). The recipient strain YYM4 is azole hypersensitive, as it lacks the endogenous pumps Pdr5p and Snq2p, the closest homologues of Cdr1p and Cdr2p. Extracts from strain YYM4 harbouring a multicopy plasmid with *CDR1* or *CDR2* or the empty vector control were analysed for protein levels using polyclonal Cdr1p or Cdr2p antisera respectively [Fig. 3A, lane 1 (Cdr1p), lane 5 (Cdr2p)]. Both ABC efflux pumps were expressed in yeast with an apparent mobility corresponding to their predicted molecular mass (Fig. 3A). Further, to show proper subcellular localization of Cdr1p and

Cdr2p in yeast, we also tagged the corresponding genes at their C-terminus with green fluorescent protein (GFP) as described in *Experimental procedures*. Fluorescence microscopy revealed that both GFP-tagged transporters localized properly to the plasma membrane (Fig. 3B, centre for Cdr1p-GFP and right for Cdr2p-GFP), as is obvious from the rim-like staining of the cells (Fig. 3B). Remarkably, however, immunoblotting with an anti-GFP antibody demonstrated that protein levels of Cdr1p-GFP were at least 50-fold higher than those of Cdr2p-GFP (Fig. 3A). Unspecific cross-reactions to Cdr1p, Cdr2p and GFP antibodies confirmed equal protein loading on each immunoblot.

To test the functionality of Cdr1p and Cdr2p in yeast, we performed growth inhibition assays using antifungal drugs such as azoles and terbinafine (Fig. 3C). Both Cdr1p and Cdr2p conferred hyper-resistance to these antifungal drugs when compared with the isogenic empty vector control YYM4, albeit to a different extent. Although Cdr1p-expressing cells showed elevated azole and terbinafine tolerance, YYM4 cells expressing Cdr2p showed a much less pronounced drug resistance (Fig. 3C). The same was also true for the GFP fusion proteins (Fig. 3C), demonstrating that the Cdr-GFP variants are functional in yeast.

Cdr2p confers resistance to caspofungin in yeast and C. albicans

Because the mRNA levels of *CDR1* and *CDR2* in the clinical isolates implied a possible direct or indirect contribution of either Cdr1p and Cdr2p to caspofungin resistance, we analysed *CDR1*- and *CDR2*-expressing yeast strains for their caspofungin MICs (Table 1, Fig. 4). Hence, YYM4 cells harbouring *CDR1*, *CDR2* or the GFP-tagged variant of each pump were tested on plates with caspofungin ranging from 0.1 to 1 μg ml^{-1} (80–800 nM). Strikingly, YYM4 cells expressing *CDR1* or *CDR2* grew much better on caspofungin plates than the isogenic empty vector control (Fig. 4). The Cdr1p-expressing strain failed to grow at 0.6 μg ml^{-1}, whereas expression of Cdr2p rendered cells resistant to caspofungin concentrations above 1 μg ml^{-1}. Unexpectedly, both GFP-tagged variants conferred reduced caspofungin resistance. Thus, GFP tagging impairs Cdr1p and Cdr2p with respect to caspofungin resistance (Fig. 4), whereas no significant differences were observed on azole or terbinafine plates (Fig. 3C). Hence, GFP fused to the C- terminus of Cdr1p or Cdr2p allows for proper plasma membrane localization, but selectively interferes with caspofungin transport. The caspofungin resistance phenotypes were observed in selective medium lacking uracil at pH 5.5 (Fig. 4), as well as in rich medium with pH 6.8 or in a different genetic background of *S. cerevisiae* lacking eight plasma membrane ABC transporters (data not shown).

To demonstrate that constitutive Cdr2p overexpression can also lead to elevated caspofungin resistance in *C. albicans*, we used the strains CAI4 (data not shown) and CAF4-2 to put *CDR2* expression under the control of the strong *ACT1* promoter. Thus, *CDR2* expression is constitutive in strain CAF4-2-ACT1-CDR2, leading to Cdr2p levels that are only detectable in the overexpressing strain, but not in the isogenic control strain CAF4-2C (Fig. 5A). Most importantly, CAF4-2-ACT1-CDR2 cells showed a dramatic increase in caspofungin resistance compared

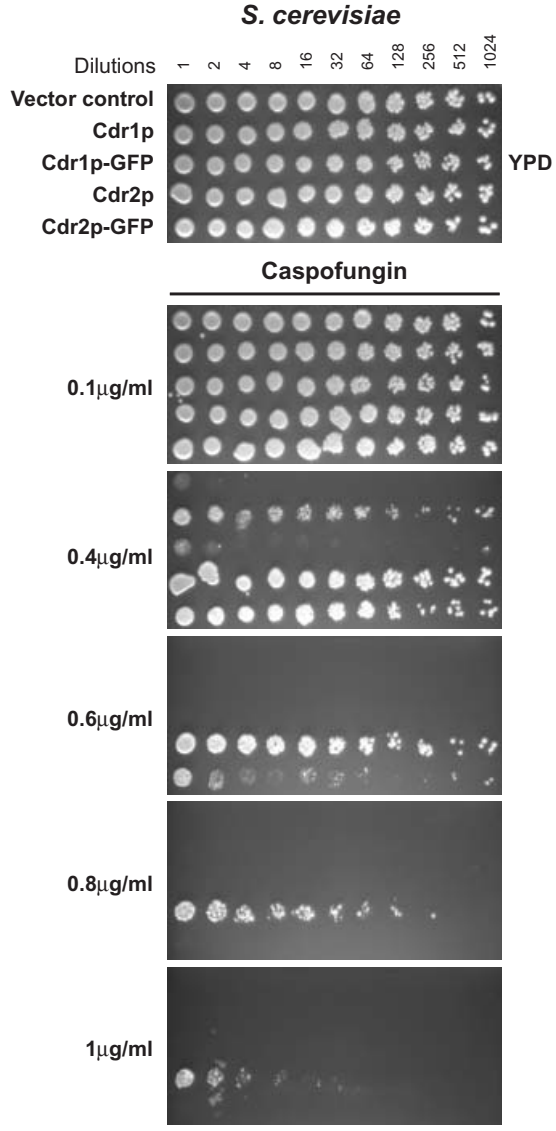

Fig. 4. Cdr2p confers resistance to caspofungin *in vitro*. Drug sensitivity assays of YYM4 *S. cerevisiae* cells carrying plasmids pDS243 (*CDR1*), pDS246 (*CDR2*), the GFP-tagged versions of both proteins or Yep24 as the empty vector control. Aliquots (5 μl) of a 0.1 OD_{600} culture (≈ 5000 cells per spot) were spotted onto drug-containing agar plates, along with twofold serial dilutions ranging from 1:2 to 1:1024. Colony growth was detected after a 72 h incubation period at 30°C.

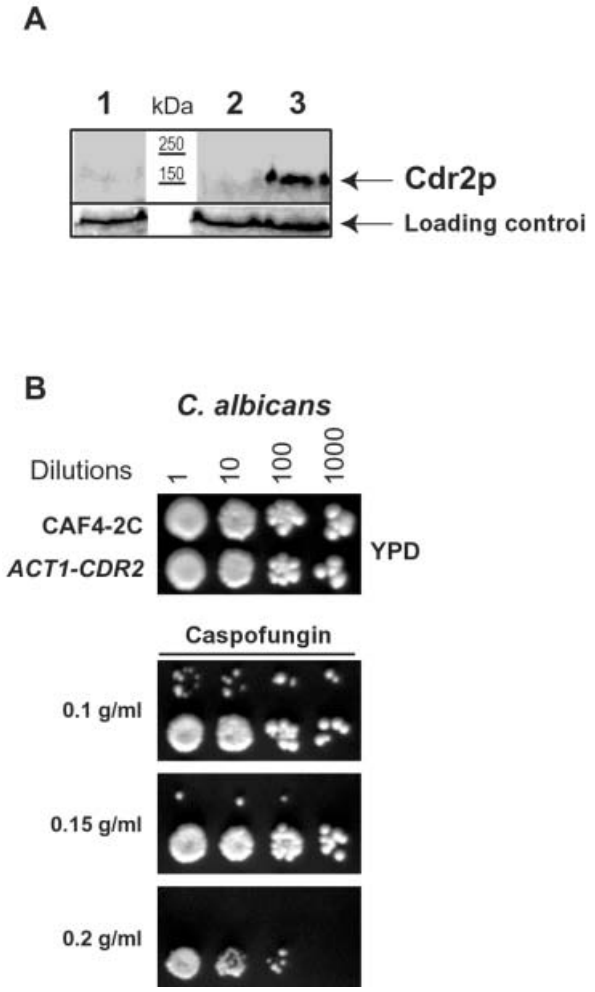

Fig. 5. Constitutive Cdr2p expression in *C. albicans* confers caspofungin resistance.
A. Immunoblot of cell-free extracts from the CAF4-2C control strain and CAF4-2-ACT1-CDR2 cells. Equivalents of 1 OD_{600} per lane were fractionated in 7% SDS-PAGE gels. Immunoblotting to nitrocellulose and subsequent immunodetection used polyclonal anti-Cdr2p (1:2500) serum. Lane 1, negative control of cell extract from the homozygous Δ*cdr2*/Δ*cdr2* deletion strain DSY653; lane 2, control strain CAF4-2 *ura3::imm434*/*URA3 CDR2*/*CDR2*; lane 3, extracts from strain CAF4-2-ACT1-CDR2 (*ura3::imm434*/*URA3 CDR2*/*ACT1-CDR2*) containing *ACT1* promoter-driven Cdr2p levels. A cross-reaction to Cdr2p antibodies served as internal loading control.
B. Agar plate drug resistance assay of CAF4-2C control cells and CAF4-2-ACT1-CDR2 cells; 0.1 OD_{600} equivalents of cells as well as three 10-fold serial dilutions were spotted onto YPD plates containing the indicated concentrations of caspofungin. Colony growth was monitored after a 72 h incubation at 30°C.

with the control strain CAF4-2C, demonstrating that Cdr2p is also able to confer candin tolerance when constitutively overexpressed in *C. albicans* from another promoter (Fig. 5B).

Relevant antifungal resistance phenotypes were then quantified using the standard NCCLS microdilution M27-A protocol (Table 1). However, although the NCCLS protocol is suitable and recommended for clinical MIC testing of standard antifungals, the results for caspofungin are unfortunately not reliable (Stone *et al*., 2002). In agreement with that, we also observed a discrepancy between MICs determined by the NCCLS method and the semi-quantitative but fully reproducible agar plate resistance assay (Table 1). The NCCLS method failed to indicate significant differences between caspofungin MICs of the reference strain and the clinical isolates. In contrast, the agar plate assays suggested up to fourfold differences in clinical isolates, and up to 10-fold increased MICs in isogenic yeast strains expressing Cdr1p and Cdr2p (Table 1). Moreover, the plate assays were highly reproducible in yeast and *C. albicans*, whereas the NCCLS method was only reproducible and thus useful for azoles and other antifungals, but not for caspofungin (Stone *et al*., 2002). In summary, our data show for the first time that Cdr2p and, to a lesser extent, Cdr1p confer resistance to caspofungin, the newest class of antifungal drugs. Importantly, our results imply that the appearance of clinical caspofungin resistance may be just a matter of time, and is perhaps caused by one of the major resistance mechanisms, the ATP-dependent efflux by ABC transporters.

Discussion

Caspofungin acetate, the semi-synthetic amine derivative of the natural product pneumocandin B_O, is a cyclic hexapeptide with fatty acyl side-chains. This new class of antifungal agents is effective even against azole-resistant clinical isolates (Maesaki *et al*., 2000; Bachmann *et al*., 2002) with elevated drug transporter expression, as shown by MIC testing against micafungin (Maesaki *et al*., 2000) or caspofungin (Bachmann *et al*., 2002). Here, we show that clinical azole-resistant *C. albicans* isolates display high caspofungin MICs. Moreover, we show that functional expression of *CDR2* and, to a much lesser extent, of *CDR1* in yeast leads to increased MIC values against caspofungin. This is the first report of elevated candin MICs for *C. albicans* isolates, and the first demonstration that fungal ABC transporters may mediate efflux-based candin resistance.

Our results demonstrate that the substrate specificity of the Cdr2p ABC efflux pump includes the most recently introduced antifungal caspofungin, a member of the echinocandins. However, previous studies on other *C. albicans* strains lacking Cdr1p and/or Cdr2p failed to observe significant changes in MIC values compared with the isogenic parent strain (Bachmann *et al*., 2002). Moreover, no severe MIC differences were seen in sequential *C. albicans* isolates derived from a single patient after antifungal treatment. In agreement with these data, our measurements of MICs using the NCCLS protocol revealed no dramatic differences in MICs between the azole-

resistant isolates and the reference strain (Table 1). In sharp contrast, growth inhibition assays on agar plates clearly show a higher susceptibility of the reference strain ATTC44374 when compared with the clinical isolates. The NCCLS protocol M27-A therefore seems not to be the most suitable protocol for caspofungin MIC testing (Nelson *et al*., 1997; Lozano-Chiu *et al*., 1999; Pfaller *et al*., 2001). Misleading results from the well-described NCCLS protocol M27-A for caspofungin might explain reports showing unchanged caspofungin MICs for azole-susceptible and -resistant *Candida* isolates (Maesaki *et al*., 2000; Bachmann *et al*., 2002). Increased caspofungin MICs for otherwise azole-resistant *C. albicans* isolates, compared with the ATCC44374 reference strain, are only detectable by growth inhibition assays on agar plates. In addition, a defined breakpoint for determining clinically relevant caspofungin MICs has not been determined as yet. Hence, our data also suggest a need for new standardized protocols allowing for candin susceptibility testing of clinical isolates.

In our studies, we were unable to include control strains such as sequential, congenic azole-susceptible isolates to verify stepwise increasing caspofungin MICs. We addressed this pitfall in several ways. First, we tested a *C. albicans* laboratory reference strain, which had never been exposed to antifungal drugs. Secondly, we pursued a functional expression approach of Cdr1p and Cdr2p in yeast, a genetically defined model organism originally exploited for the molecular identification of Cdr1p and Cdr2p (Sanglard *et al*., 1995; 1997). This approach, like a similar one reported elsewhere (Nakamura *et al*., 2001), shows that one can produce a useful yeast tester strain to study individual contributions of certain resistance genes to compound cross-resistance phenotypes. In this way, we demonstrate that Cdr2p is more active in conferring caspofungin resistance, whereas azole resistance appears to be less pronounced. Cdr1p-mediated azole and terbinafine resistance was always higher than Cdr2p-mediated resistance to the same drugs, probably because of higher levels of Cdr1p. Conversely, Cdr2p-mediated caspofungin resistance was drastically higher than for Cdr1p, despite its 50-fold lower protein levels.

The reasons for the lower expression level of Cdr2p in yeast are unclear at present. The *CDR2* promoter might be weaker in yeast than the *CDR1* promoter, although *CDR2* is also weakly expressed in various *Candida* spp. (Sanglard *et al*., 1997). We cannot rule out at the moment the possibility that a different codon usage might reduce *CDR2* expression, as it results in three residue changes (S21L, S632L and S788L) in yeast-expressed Cdr2p. Although unlikely, the amino acid changes could confer increased caspofungin efflux capacity on Cdr2p in yeast. Further experiments and mutational studies are required to resolve this interesting issue. In any case, lipopeptides are also effluxed by other fungal ABC pumps such as the cilofungin transporter AfuMdr1p from *Aspergillus fumigatus* (Tobin *et al*., 1997) or the yeast Ste6p mating pheromone transporter (Kuchler *et al*., 1989; McGrath and Varshavsky, 1989).

A comparison of our data with published reports on echinocandin-resistant yeasts (Kurtz *et al*., 1996) reveals that high caspofungin MICs can be linked for the first time to ABC transporter overexpression. Although one would predict that mutations in the echinocandin target gene Ca*FKS1* (Douglas *et al*., 1997; Mio *et al*., 1997) as well as increased ABC efflux pumps may contribute to elevated MICs in the clinical *C. albicans* isolates *in vivo*, our data clearly indicate that Cdr2p alone is sufficient to confer caspofungin tolerance. Indeed, the polymerase chain reaction (PCR) cloning and sequence analysis of the *CaFKS1* genes (Douglas *et al*., 1997; Mio *et al*., 1997) isolated from the clinical isolates 'B', 'f' and 'i' used in this study failed to identify any relevant target mutations that could explain the high caspofungin MICs observed *in vitro* (data not shown).

Earlier studies suggested that CDR-mediated drug efflux seems not to be involved in resistance to the semisynthetic pneumocandin L-733,560 or caspofungin respectively (Kurtz *et al*., 1996; Bachmann *et al*., 2002). However, we do not know at present whether or not L-733,560, a compound related to caspofungin, is also a substrate for Cdr2p. Nevertheless, we show a direct correlation between expression of Cdr2p and elevated caspofungin resistance, both in a heterologous host and *C. albicans*. Furthermore, echinocandin-resistant isolates created by stepwise selection *in vitro* remained susceptible to other antifungals, including AmB, fluconazole or itraconazole (Kurtz *et al*., 1996). Interestingly, we can show that certain azole-resistant clinical isolates of *C. albicans* ('B' and 'i') display high resistance to ketoconazole (Fig. 1B), while being less resistant to caspofungin than 'f' (Fig. 2). The latter displays the lowest azole resistance (Fig. 1B), but highest resistance to caspofungin (Fig. 2). These data are entirely consistent with the previous results showing fluconazole or itraconazole susceptibility of echinocandin-resistant yeast cells (Kurtz *et al*., 1996). Therefore, the clinical use of antifungal drugs with distinct mechanisms of action might generate a therapeutic advantage. Indeed, a combination of terbinafine and antifungal azoles in clinical therapy results in synergy in antifungal efficacy and thus improved therapies (Ryder and Leitner, 2001).

Interestingly, Sbe2p, a Golgi-resident protein involved in cell wall synthesis, has recently also been linked to caspofungin resistance. Although the mechanism is unknown, it does not involve ATP-dependent candin efflux (Osherov *et al*., 2002). Cilofungin, a predecessor of caspofungin (Pfaller *et al*., 1989), causes changes in plasma mem-

brane and cell wall composition, with a dramatic decrease in cell wall glucan. In addition, cilofungin causes a sharp decrease in plasma membrane ergosterol, as well as an increase in chitin and mannan (Pfaller *et al*., 1989). The increase in cell wall chitin and mannan may therefore provide a salvage pathway to bypass inhibition of the glucan synthase (Popolo *et al*., 2001). Likewise, echinocandins interfere with the plasma membrane properties (Ko *et al*., 1994), and ABC transporters such as Cdr1p and Cdr2p have been implicated in changes in plasma membrane permeability (Kohli *et al*., 2002; Smriti *et al*., 2002). Thus, we propose that clinical caspofungin resistance *in vivo* may perhaps be the consequence of a combination of altered plasma membrane features, candin target gene mutations, as well as Cdr2p-mediated active drug efflux. Taken together, our studies demonstrate for the first time Cdr2p-mediated caspofungin resistance in the heterologous model system *S. cerevisiae*. Moreover, the constitutive overexpression of *CDR2* driven by the *ACT1* promoter in *C. albicans* laboratory strains such as CAI4 (data not shown) and CAF4-2 (Fig. 5) unequivocally demonstrates that Cdr2p can contribute to caspofungin tolerance *in vivo*. These results further support the notion that elevated caspofungin MICs (Table 1) exhibited in clinical isolates 'B', 'f' and 'i' are, at least in part, caused by increased expression of Cdr2p (Fig. 1A). The verification of a therapeutic relevance will require screening of additional clinical isolates, particularly sequential congenic isolates, as soon as reliable protocols for caspofungin MIC testing become available. To come full circle, our results also suggest to implement resistance monitoring to echinocandins, which have been as yet effective antifungal drugs.

Experimental procedures

Media, fungal strains and culture conditions

Rich media (YPD for both *S. cerevisiae* and *C. albicans*, Sabouraud only for *C. albicans*) and selective media supplemented with auxotrophic components to maintain plasmids were prepared as described previously (Kaiser *et al*., 1994). All media ingredients were purchased from Difco, and strains were grown routinely at 30°C. The yeast recipient strain used for functional expression studies was *S. cerevisiae* YYM4 (*MAT***a** Δ*snq2*::hisG Δ*pdr5*::*TRP1 ura3-52 his3-*Δ*200 leu2-*Δ*1 trp1-*Δ*63 lys2–801*^{amb} *ade2*-101^{oc}) (Egner *et al*., 1998). *Candida albicans* strains were the ATCC44374 wild type as a reference strain, as well as azole-resistant clinical isolates of *C. albicans* referred to as 'B', 'f' and 'i'. These strains have been collected at the General Hospital of Vienna since the beginning of 1996. Identification of clinical isolates was done by assimilation assays (APID32C, bioMérieux). Morphological analysis on rice extract agar used a standard microbiology laboratory protocol (Rippon, 1988). Further, the laboratory strains *C. albicans* CAF4-2 and CAI4 (Sanglard *et al*., 1996), both of which are carrying the homozygous mutation *ura3*::*imm434/ura3*::*imm434*, were used and propagated in YPD supplemented with 80 μg ml^{-1} uridine.

Plasmids and DNA manipulations

Cdr1p and Cdr2p were expressed from their own promoters in YYM4 using the multicopy plasmids pDS243 (*CDR1*) and pDS246 (*CDR2*) respectively (Sanglard *et al*., 1996). Transformation of yeast was done by the lithium acetate method (Kaiser *et al*., 1994) or by electroporation (Grey and Brendel, 1992). The tagging of *CDR1* and *CDR2* with the green fluorescent protein GFP-S65T used a PCR-based technique and plasmid pFA6a-GFPS65T-HIS3-MX6 (Wach *et al*., 1997). Integration of the tagging cassette just before the *CDR1*/*CDR2* stop codons was done in YYM4 by *in vivo* cloning using plasmids pDS243 (*CDR1*) or pDS246 (*CDR2*) respectively.

A *C. albicans* strain overexpressing *CDR2* under the actin promoter was constructed in the CAF4-2 and CAI4 genetic backgrounds as follows. The first 1500 bp of *CDR2* was amplified by PCR using the high-fidelity PCR kit (Roche). The primer pair CDR2-XhoIfw (5′-AAACTCGAGAAAAACATATGAGTACTGCAAACACGTCTTTGTCGCAAC-3′) and CDR2-STOPBamHIrev (5′-AAAGGATCCATTAAGGTCTTGTATTGTTGGATTGTTTGCCAACATG-3′) introduced a *Xho*I site at nucleotide position −13 of the *CDR2* open reading frame (ORF) and a stop codon followed by a *Bam*HI site at nucleotide position +1449 of *CDR2*. The PCR fragment was subcloned into pGEM-T (Promega) and verified by DNA sequencing using the BigDye Terminator cycle sequencing kit version 3.0 and the ABI Prism sequencing system 310 (Applied Biosystems). The fragment was subsequently cloned into the *Xho*I–*Bam*HI sites of pAU34 (Dieterich *et al*., 2002), yielding pAU34-ACT1-CDR2. The *Hin*dIII site at nucleotide position +939 in *CDR2* was used to linearize the plasmid before transformation of the linear fragment into CAI4 and CAF4-2 (*ura3*::*imm434/ura3*::*imm434*) cells using the LiAc method (Gietz and Woods, 2001). Transformants were selected on synthetic complete medium lacking uracil, and correct genomic integration of the plasmid-derived *ACT1* promoter fused to the *CDR2* ORF was verified by PCR using the primer pair CaACT1-161fw (5′-TTCTCACCAGGATTTATTGCC-3′) and CDR21724rev (5′-TCCACAATTGGTCTAGCTTCAT-3′). Several independent transformants were analysed for Cdr2p expression, and one representative transformant of CAF4-2 was used for further analysis. The appropriate heterozygous *ura3/URA3* control strain CAF4-2C not overexpressing Cdr2p was constructed by genomically integrating the *Nde*I-linearized empty plasmid pAU34 (Dieterich *et al*., 2002).

Microdilution assays to determine MIC values

Microdilution was performed according to the NCCLS guidelines document M27-A. Caspofungin acetate and fluconazole were diluted in sterile water, whereas ketoconazole and itraconazole were dissolved in dimethyl sulphoxide (DMSO). All tests were done in 96-well round-bottomed microtitre plates (Greiner). Yeast suspensions of McFarland 0.5 corresponding

to 1–5 × 10^6 cells ml^{-1} were prepared in 0.85% NaCl and diluted with RPMI-1640 medium adjusted to 2% glucose and buffered to pH 7.0 with 0.165 M MOPS (Sigma). The final cell count approximated 0.5–2.5 × 10^3 cells ml^{-1}. The plates were incubated for 48 h at 35°C, and growth was monitored after 24 h and 48 h. The turbidities to read MICs were determined spectrophotometrically at 630 nm using a MR5000/7000 microplate reader (Dynatech Laboratories). End-points were calculated as minimal inhibitory concentration 80 (MIC), corresponding to the lowest drug concentration that inhibited growth equal to or greater than 80% of the control. Final concentrations of caspofungin acetate ranged from 126 to 0.12 mg l^{-1}, for fluconazole 64–0.06 mg l^{-1} and 16–0.01 mg l^{-1} for all other azoles.

Agar plate drug resistance assays

Fresh overnight cultures were inoculated into rich or selective medium and grown to an optical density (OD_{600}) of about 1. Cultures were diluted to an OD_{600} of about 0.2 and spotted, along with serial dilutions of 1:10, 1:100 and 1:1000, on drug-containing agar plates. These plates were prepared by adding the selected compounds from stock solutions to the molten agar equilibrated at 50°C (Bissinger and Kuchler, 1994). Cell growth was monitored after 48 h or 72 h incubation at 30°C. Caspofungin was prepared as a 5 mg ml^{-1} (4.12 mM) stock directly from commercially available Cancidas™ (Merck) caspofungin acetate by dissolving in sterile distilled water. Stock solutions of 10 mM fluconazole (Pfizer), 0.5 mM ketoconazole (Sigma), 0.5 mM itraconazole (Janssen Pharmaceuticals) and 10 mM terbinafine (Novartis) were prepared in DMSO and stored at –20°C.

Northern blot analysis

Total RNA was prepared from *C. albicans* cultures grown in Sabouraud medium to the exponential growth phase. Cells equivalent to 10 OD_{600} were harvested, and RNA was extracted by the hot acidic phenol method as described elsewhere (Schmitt *et al*., 1990). Aliquots of 20 μg of total RNA were separated in glyoxal gels exactly as described previously (Wolfger *et al*., 1997) and subsequently blotted to Hybond-N™ nylon filters. As loading controls, the ribosomal RNA bands were stained with methylene blue after filter transfer. Detection of *CDR1* or *CDR2* mRNAs used PCR-derived probes corresponding to nucleotides (nt) 95–576 and nt 10–468 respectively. Hybridization of blots was performed at 65°C overnight.

Preparation of cell-free extracts and immunoblotting

Saccharomyces cerevisiae cells of strain YYM4 containing plasmids pDS243 (*CDR1*), pDS246 (*CDR2*) or the empty vector control Yep24 were grown overnight in selective medium. *C. albicans* CAF4-2C control cells or CAF4-2-ACT1-CDR2 were grown in YPD, diluted to an OD_{600} of 0.2 and then grown to the exponential growth phase, corresponding to an OD_{600} of 1–2. Cells equivalent to 5 OD_{600} were lysed with 250 μl of YEX lysis buffer (1.85 M NaOH, 7.5% β-mercaptoethanol), incubated for 10 min on ice and precipitated with 250 μl of 50% (w/w) trichloracetic acid for 10 min on ice. The protein precipitate was dissolved in 100 μl of sample buffer (40 mM Tris-HCl, p6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, 0.1 g l^{-1} bromophenol blue, supplemented with 1% β-mercaptoethanol and 10% 1 M Tris-base). Aliquots of 10 or 40 μl of cell-free extracts, corresponding to 0.5 (Cdr1p and Cdr2p antiserum) or 2 OD_{600} (GFP antibody) were separated by SDS-PAGE in 7.5% gels and blotted to nitrocellulose membranes. Immunodetection used polyclonal antisera against Cdr1p or Cdr2p (kindly provided by D. Sanglard) or a commercially available monoclonal anti-GFP antibody (Roche). The dilutions were 1:2500 for the antisera against Cdr1p and Cdr2p, and the GFP antibody was diluted 1:1000. Proteins on immunoblots were visualized using the ECL™ chemiluminescence detection system (Amersham).

Fluorescence microscopy

To view GFP fluorescence, yeast cells were streaked on SC –URA medium supplemented with adenine at a final concentration of 40 mg l^{-1} to minimize vacuolar autofluorescence caused by adenine metabolites. Single colonies were picked and resuspended in 1 ml of water containing 1 μg ml^{-1} 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) to stain nuclear DNA for 10 min at 30°C. After a brief wash with water, cells were viewed under the Axiovert 135 fluorescence microscope (Zeiss) equipped with a fluorescein isothiocyanate (FITC), DAPI and DIC filter set. Pictures were taken using a Quantix CCD camera (Roper Scientific) and processed with the IP-LAB software (Spectra Services); final adjustments were done with the PHOTOSHOP software (Adobe).

Acknowledgements

We thank Steffen Rupp, Christophe D'enfert and Dominique Sanglard for the generous gift of reagents, strains and plasmids. The critical reading of the manuscript and helpful discussions by Bettina Bauer, Christoph Schüller, Yasmine Mamnun and Hubert Wolfger are highly appreciated. This work was supported by a grant from the 'Hygiene-Fonds' of the Medical Faculty of the University of Vienna to K.K. and B.W. and, in part, by grants from the Austrian Science Foundation (P-12661-BIO and P-15934-B08) and Novartis Pharma, Inc. to K.K., as well as COST action B16.

References

Abruzzo, G.K., Flattery, A.M., Gill, C.J., Kong, L., Smith, J.G., Pikounis, V.B., *et al.* (1997) Evaluation of the echinocandin antifungal MK-0991 (L-743,872): efficacies in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. *Antimicrob Agents Chemother* **41:** 2333–2338.

Bachmann, S.P., Patterson, T.F., and Lopez-Ribot, J.L. (2002) *In vitro* activity of caspofungin (MK-0991) against *Candida albicans* clinical isolates displaying different mechanisms of azole resistance. *J Clin Microbiol* **40:** 2228–2230.

Bartizal, K., Gill, C.J., Abruzzo, G.K., Flattery, A.M., Kong, L., Scott, P.M., *et al.* (1997) *In vitro* preclinical evaluation

studies with the echinocandin antifungal MK-0991 (L-743,872). *Antimicrob Agents Chemother* **41:** 2326–2332.

Bastert, J., Schaller, M., Korting, H.C., and Evans, E.G. (2001) Current and future approaches to antimycotic treatment in the era of resistant fungi and immunocompromised hosts. *Int J Antimicrob Agents* **17:** 81–91.

Bauer, B.E., Wolfger, H., and Kuchler, K. (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochim Biophys Acta* **1461:** 217–236.

Bissinger, P.H., and Kuchler, K. (1994) Molecular cloning and expression of the *Saccharomyces cerevisiae STS1* gene product. A yeast ABC transporter conferring mycotoxin resistance. *J Biol Chem* **269:** 4180–4186.

Chiou, C.C., Groll, A.H., and Walsh, T.J. (2000) New drugs and novel targets for treatment of invasive fungal infections in patients with cancer. *Oncologist* **5:** 120–135.

De Backer, M.D., Ilyina, T., Ma, X.J., Vandoninck, S., Luyten, W.H., and Vanden Bossche, H. (2001) Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. *Antimicrob Agents Chemother* **45:** 1660–1670.

Denning, D.W. (2002) Echinocandins: a new class of antifungal. *J Antimicrob Chemother* **49:** 889–891.

Dieterich, C., Schandar, M., Noll, M., Johannes, F.J., Brunner, H., Graeve, T., and Rupp, S. (2002) *In vitro* reconstructed human epithelia reveal contributions of *Candida albicans EFG1* and *CPH1* to adhesion and invasion. *Microbiology* **148:** 497–506.

Douglas, C.M., Marrinan, J.A., Li, W., and Kurtz, M.B. (1994a) A *Saccharomyces cerevisiae* mutant with echinocandin-resistant 1,3-beta-D-glucan synthase. *J Bacteriol* **176:** 5686–5696.

Douglas, C.M., Foor, F., Marrinan, J.A., Morin, N., Nielsen, J.B., Dahl, A.M., *et al.* (1994b) The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-beta-D-glucan synthase. *Proc Natl Acad Sci USA* **91:** 12907–12911.

Douglas, C.M., D'Ippolito, J.A., Shei, G.J., Meinz, M., Onishi, J., Marrinan, J.A., *et al.* (1997) Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-beta-D-glucan synthase inhibitors. *Antimicrob Agents Chemother* **41:** 2471–2479.

Egner, R., Rosenthal, F.E., Kralli, A., Sanglard, D., and Kuchler, K. (1998) Genetic separation of FK506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. *Mol Biol Cell* **9:** 523–543.

Ellis, M. (2002) Invasive fungal infections: evolving challenges for diagnosis and therapeutics. *Mol Immunol* **38:** 947–957.

Gietz, R.D., and Woods, R.A. (2001) Genetic transformation of yeast. *Biotechniques* **30:** 816–820, 822–816, 828 (passim).

Grey, M., and Brendel, M. (1992) A ten-minute protocol for transforming *Saccharomyces cerevisiae* by electroporation. *Curr Genet* **22:** 335–336.

Heimark, L., Shipkova, P., Greene, J., Munayyer, H., Yarosh-Tomaine, T., DiDomenico, B., *et al.* (2002) Mechanism of azole antifungal activity as determined by liquid chromatographic/mass spectrometric monitoring of ergosterol biosynthesis. *J Mass Spectrom* **37:** 265–269.

Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics. A Laboratory Course Manual*. New York: Cold Spring Harbor Laboratory Press.

Ko, Y.T., Ludescher, R.D., Frost, D.J., and Wasserman, B.P. (1994) Use of cilofungin as direct fluorescent probe for monitoring antifungal drug–membrane interaction. *Antimicrob Agents Chemother* **38:** 1378–1385.

Kohli, A., Smriti, Mukhopadhyay, K., Rattan, A., and Prasad, R. (2002) *In vitro* low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. *Antimicrob Agents Chemother* **46:** 1046–1052.

Kuchler, K., Sterne, R.E., and Thorner, J. (1989) *Saccharomyces cerevisiae STE6* gene product: a novel pathway for protein export in eukaryotic cells. *EMBO J* **8:** 3973–3984.

Kurtz, M.B., and Douglas, C.M. (1997) Lipopeptide inhibitors of fungal glucan synthase. *J Med Vet Mycol* **35:** 79–86.

Kurtz, M.B., Abruzzo, G., Flattery, A., Bartizal, K., Marrinan, J.A., Li, W., *et al.* (1996) Characterization of echinocandin-resistant mutants of *Candida albicans*: genetic, biochemical, and virulence studies. *Infect Immun* **64:** 3244–3251.

Louie, A., Kaw, P., Banerjee, P., Liu, W., Chen, G., and Miller, M.H. (2001) Impact of the order of initiation of fluconazole and amphotericin B in sequential or combination therapy on killing of *Candida albicans in vitro* and in a rabbit model of endocarditis and pyelonephritis. *Antimicrob Agents Chemother* **45:** 485–494.

Lozano-Chiu, M., Nelson, P.W., Paetznick, V.L., and Rex, J.H. (1999) Disk diffusion method for determining susceptibilities of *Candida spp*. to MK-0991. *J Clin Microbiol* **37:** 1625–1627.

McGrath, J.P., and Varshavsky, A. (1989) The yeast *STE6* gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* **340:** 400–404.

Maesaki, S., Hossain, M.A., Miyazaki, Y., Tomono, K., Tashiro, T., and Kohno, S. (2000) Efficacy of FK463, a (1,3)-beta-D-glucan synthase inhibitor, in disseminated azole-resistant *Candida albicans* infection in mice. *Antimicrob Agents Chemother* **44:** 1728–1730.

Mazur, P., Morin, N., Baginsky, W., el-Sherbeini, M., Clemas, J.A., Nielsen, J.B., and Foor, F. (1995) Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. *Mol Cell Biol* **15:** 5671–5681.

Mio, T., Adachi-Shimizu, M., Tachibana, Y., Tabuchi, H., Inoue, S.B., Yabe, T., *et al.* (1997) Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae GSC1*/*FKS1* and its involvement in beta-1,3-glucan synthesis. *J Bacteriol* **179:** 4096–4105.

Nakamura, K., Niimi, M., Niimi, K., Holmes, A.R., Yates, J.E., Decottignies, A., *et al.* (2001) Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. *Antimicrob Agents Chemother* **45:** 3366–3374.

Nelson, P.W., Lozano-Chiu, M., and Rex, J.H. (1997) *In vitro* growth-inhibitory activity of pneumocandins L-733,560 and L-743,872 against putatively amphotericin B- and fluconazole-resistant *Candida* isolates: influence of assay conditions. *J Med Vet Mycol* **35:** 285–287.

Osherov, N., May, G.S., Albert, N.D., and Kontoyiannis, D.P. (2002) Overexpression of Sbe2p, a Golgi protein, results

in resistance to caspofungin in *Saccharomyces cerevisiae*. *Antimicrob Agents Chemother* **46:** 2462–2469.

Petranyi, G., Ryder, N.S., and Stütz, A. (1984) Allylamine derivatives: new class of synthetic antifungal agents inhibiting fungal squalene epoxidase. *Science* **224:** 1239–1241.

Pfaller, M., Riley, J., and Koerner, T. (1989) Effects of cilofungin (LY121019) on carbohydrate and sterol composition of *Candida albicans*. *Eur J Clin Microbiol Infect Dis* **8:** 1067–1070.

Pfaller, M.A., Messer, S.A., Mills, K., Bolmstrom, A., and Jones, R.N. (2001) Evaluation of E-test method for determining caspofungin (MK-0991) susceptibilities of 726 clinical isolates of *Candida* species. *J Clin Microbiol* **39:** 4387–4389.

Popolo, L., Gualtieri, T., and Ragni, E. (2001) The yeast cell-wall salvage pathway. *Med Mycol* **39:** 111–121.

Powles, M.A., Liberator, P., Anderson, J., Karkhanis, Y., Dropinski, J.F., Bouffard, F.A., *et al.* (1998) Efficacy of MK-991 (L-743,872), a semisynthetic pneumocandin, in murine models of *Pneumocystis carinii*. *Antimicrob Agents Chemother* **42:** 1985–1989.

Prasad, R., Dewergifosse, P., Goffeau, A., and Balzi, E. (1995) Molecular cloning and characterization of a novel gene of *Candida albicans*, *CDR1*, conferring multiple resistance to drugs and antifungals. *Curr Genet* **27:** 320–329.

Rippon, J.W. (1988) *Medical Mycology*, Philadelphia: W.B. Saunders.

Ryder, N.S. (1999) Activity of terbinafine against serious fungal pathogens. *Mycoses* **42:** 115–119.

Ryder, N.S., and Leitner, I. (2001) Synergistic interaction of terbinafine with triazoles or amphotericin B against *Aspergillus* species. *Med Mycol* **39:** 91–95.

Ryder, N.S., Wagner, S., and Leitner, I. (1998) *In vitro* activities of terbinafine against cutaneous isolates of *Candida albicans* and other pathogenic yeasts. *Antimicrob Agents Chemother* **42:** 1057–1061.

Sanglard, D., and Odds, F.C. (2002) Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis* **2:** 73–85.

Sanglard, D., Kuchler, K., Ischer, F., Pagani, J.L., Monod, M., and Bille, J. (1995) Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother* **39:** 2378–2386.

Sanglard, D., Ischer, F., Monod, M., and Bille, J. (1996) Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob Agents Chemother* **40:** 2300–2305.

Sanglard, D., Ischer, F., Monod, M., and Bille, J. (1997) Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. *Microbiology* **143:** 405–416.

Schmitt, M.E., Brown, T.A., and Trumpower, B.L. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res* **18:** 3091–3092.

Smriti, Krishnamurthy, S., Dixit, B.L., Gupta, C.M., Milewski, S., and Prasad, R. (2002) ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. *Yeast* **19:** 303–318.

Stone, E., Fung, H.B., and Kirschenbaum, H.L. (2002) Caspofungin: an echinocandin antifungal agent. *Clin Therapeut* **24:** 351–377.

Tobin, M.B., Peery, R.B., and Skatrud, P.L. (1997) Genes encoding multiple drug resistance-like proteins in *Aspergillus fumigatus* and *Aspergillus flavus*. *Gene* **200:** 11–23.

Wach, A., Brachat, A., Alberti-Segui, C., Rebischung, C., and Philippsen, P. (1997) Heterologous *HIS3* marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast* **13:** 1065–1075.

White, T.C., Holleman, S., Dy, F., Mirels, L.F., and Stevens, D.A. (2002) Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrob Agents Chemother* **46:** 1704–1713.

Wingard, J.R., Merz, W.G., Rinaldi, M.G., Miller, C.B., Karp, J.E., and Saral, R. (1993) Association of *Torulopsis glabrata* infections with fluconazole prophylaxis in neutropenic bone marrow transplant patients. *Antimicrob Agents Chemother* **37:** 1847–1849.

Wolfger, H., Mahé, Y., Parle-McDermott, A., Delahodde, A., and Kuchler, K. (1997) The yeast ATP-binding cassette (ABC) protein genes *PDR10* and *PDR15* are novel targets for the Pdr1 and Pdr3 transcriptional regulators. *FEBS Lett* **418:** 269–274.