The fungicide fludioxonil antagonizes fluconazole activity in the human fungal pathogen *Candida albicans*

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abbreviations:
ABC-transporter – transporters with ATP-binding cassettes
PDR – pleiotropic drug resistance
R6G – rhodamine 6G

Abstract

The fungicide fludioxonil is widely used in agriculture. Residua of this fungicide are occasionally detected in fruits and can therefore be ingested by humans.

The human fungal pathogen *Candida albicans* expresses the target of fludioxonil, Nik1p, a type III histidine kinase involved in stress response. Inhibition of yeast and hyphae growth was hardly observable after treatment of *C. albicans* SC5314 with fludioxonil. As a side effect, however, we observed a concentration-dependent induction of the expression of the genes *CDR1* and *CDR2* for ABC-transporters (ATP-binding cassette transporter). This was independent of the presence of the target of fludioxonil, as induction was also observed in a *Δnik1* deletion mutant. Deletion of *CDR1* aggravated the inhibition of germ tube formation by fludioxonil, indicating that, in the wild type, the fungicide was discharged from the cell by Cdr1p. Cdr1p is also known as a resistance factor of *C. albicans* against the commonly used antimycotic fluconazole.

Thus the effect of concurrent exposition to fludioxonil and known cargoes of ABC-transporters on their extrusion and the growth of *C. albicans* was examined. Pre-incubation with fludioxonil decreased the export rate of rhodamine 6G. The resistance to fluconazole was increased by fludioxonil, independently of Nik1p.

Therefore, exposition of *C. albicans* to fludioxonil may lead to increased resistance to fluconazole treatment.

Introduction

The oligomorphic yeast *C. albicans* can cause localized superficial infections as well as life-threatening systemic candidiasis in immunocompromised individuals. *C. albicans* infections accompany severe diseases, are hospital-acquired in the majority of cases and are the fourth most frequent cause of nosocomial sepsis. Thus they have become a major complication particularly in intensive care units (Pfaller and Diekema, 2007).

Fludioxonil is a phenylpyrrol fungicide which is widely used in agriculture, especially in the protection of grapes and berries from plant pathogenic fungi, most prominently *Botrytis cinerea*. While fludioxonil is highly toxic to some aquatic organisms, the toxicity to mammals has been low or negligible in a wide range of toxicological studies. The fungicide interacts with the osmotic stress response of filamentous fungi and yeasts by inhibiting the activity of a histidine kinase of type III (Pillonel and Meyer, 1997; Okada *et al*., 2005; Ochiai *et al*., 2002). A homologous histidine kinase (termed Nik1p) is expressed in the opportunistic human-pathogenic yeast *Candida albicans* (Alex *et al.*, 1998; Yamada-Okabe *et al.*, 1998) and is also targeted by fludioxonil (Buschart *et al.*, 2012). Furthermore, Nik1p has been implicated to be involved in the yeast-to-hyphae transition (Alex *et al.*, 1998; Yamada-Okabe *et al.*, 1999), which is a major pathogenicity factor (Calderone and Fonzi, 2001). Therefore, we investigated the effect of fludioxonil on *C. albicans*. We observed in a comprehensive gene expression analysis that fludioxonil induced the expression of the ABC-transporters Cdr1p and Cdr2p.

The overexpression of such transporters can cause a higher resistance to antimycotic drugs, as the intracellular concentration of the antimycotic is decreased (Wirsching *et al.*, 2000; Siikala *et al.*, 2010). Active transport by ABC-transporters, especially of the PDR-subfamily, is achieved by coupling ATP-hydrolysis to export (Prasad *et al.*, 1995; Sipos and Kuchler, 2006). The genome of *C. albicans* contains nine genes for PDR-subfamily ABC-transporters (according to Gaur *et al.*, 2005 and the *Candida* Genome Database (Arnaud *et al.*, 2012)) and the relevance of the transporters Cdr1p and Cdr2p in resistance to fluconazole has been studied intensively (Prasad *et al.*, 1995; Sanglard *et al.*, 1997).

Fluconazole is one of the azole-antimycotics which are used to treat systemic *C. albicans* infections, besides polyenes, echinocandins, and flucytosin. Acquisition of resistance to one or several of these antimycotics has been observed (Rex *et al.*, 1995; Fournier *et al.*, 2011). Resistance to fluconazole is usually found in less than 10 % of clinical isolates of *C. albicans*, but the proportion of fully susceptible strains is below 80 % (Sanglard and Odds, 2002; Pfaller and Diekema, 2007).

Cdr1p seems to be more important in fluconazole resistance than Cdr2p (Tsao *et al.*, 2009). Besides

fluconazole, Cdr1p is able to export human hormones, like estradiol (Krishnamurthy *et al.*, 1998a), and phospholipids (Dogra *et al.*, 1999; Smriti *et al.*, 2002; Shukla *et al.*, 2007). A higher susceptibility of a Δ*cdr1* mutant pointed to Cdr1p's activity in the efflux of terbinafine, cycloheximide, brefeldin A and fluphenazine (Sanglard *et al.*, 1996).

Constitutive overexpression of *CDR1* and *CDR2* is caused by mutations of the transcriptional regulator Tac1p (Coste *et al.*, 2004; Coste *et al.*, 2006). Transient upregulation of *CDR1* and *CDR2*, which also attenuates susceptibility to antimycotics, has been observed upon bio-film formation and heat shock, or exposure to hormones and some antimycotic compounds as well as agricultural herbicides (Krishnamurthy *et al.*, 1998b; Liu *et al.*, 2005; Schmidt *et al.*, 2008).

Hence we studied the effect of fludioxonil on the expression of *CDR1* and *CDR2* in *C. albicans*. We found the induction of the expression of *CDR1* and *CDR2* during exposition to fludioxonil to be a target-independent side-effect. Therefore we also investigated the consequences of fludioxonil exposure for the export efficiency of antimycotic compounds and for the susceptibility of *C. albicans* to these compounds.

Material and methods

Organisms and culture conditions

C. albicans isolates ATCC-10231, DSM-1577 (both obtained from DSMZ-German Collection of Microorganisms and Cell Cultures) and SC5314 (ATCC MYA-2876; Gillum *et al.*, 1984), as well as the SC5314-derived deletion mutants Δ*cdr1* (Nobile and Mitchell, 2009), Δ*cdr2* (Sanglard *et al.*, 1997), Δ*cdr1,2* (Sanglard *et al.*, 1997), Δ*tac1* (Homann *et al.*, 2009), and *Δnik1* (Alex *et al.*, 1998) were used in this study. Overnight cultures were prepared in 250 ml flasks with 50 ml YPD medium at 30 °C. Pre-cultures were prepared by diluting the overnight culture in 20 ml YNB (yeast nitrogen base without amino acids, 20 g l^{-1} glucose, 0.165 M MOPS, pH 7.2, supplemented with 20 mg l^{-1} L-histidine or L-arginine) to an OD_{620nm} of 0.2 and incubated for 3 h until they reached the exponential growth phase. All measurements of optical density were carried out by using the microtitre plate spectrophotometer µQuant (Biotek) and sample volumes of 180 µl in standard 96-well plates. Treatment with fungicides was carried out during the subsequent main culture and appropriate solvent controls were used for comparison.

Susceptibility assays

Sensitivity of *C. albicans* to antifungal compounds in liquid culture were determined in microtitre

plates (180 µl volume). 5000 cells per ml from an exponentially growing pre-culture were incubated in YNB with dilution series of the compounds at 30 °C for 24 h and growth was determined photometrically by measuring the OD_{620nm}. Combination effects of compounds were investigated using the respective compound mixtures. Experiments were conducted in triplicate cultures. Results from three independent experiments are reported here.

In addition, fluconazole sensitivity was evaluated according to the standard recommendations of EUCAST (EUCAST, 2008) using inocula containing different concentrations of fludioxonil.

Analysis of germ tube formation

C. albicans from an exponentially growing pre-culture were incubated in RPMI-1640 (buffered to pH 7 with 0.165 M MOPS) and supplemented with or without 20 µg ml^{-1} fludioxonil at 37 °C for 4 h. Every 60 min, a sample was analyzed microscopically. 300 to 1500 yeast cells were counted in each experiment and the portion of cells which had formed germ tubes was documented. Data from two independent experiments are presented.

Transcriptome analysis by microarray

C. albicans from an exponentially growing pre-culture were incubated in RPMI-1640 (buffered to pH 7 with 0.165 M MOPS) and supplemented with or without 20 µg ml^{-1} fludioxonil at 37 °C for 30 min. The cells were harvested by centrifugation at 8000 ∗ g for 2 min, and the cell pellets were shock-frozen in liquid nitrogen. Frozen pellets were suspended in 0.6 ml RLT buffer (Qiagen) and mechanically disrupted using glass beads (425 to 600 µm, Sigma). RNA was isolated on RNeasy mini columns with added DNase (Qiagen) as recommended by the manufacturer. The quality and integrity of total RNA of the samples was controlled with the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies).

Cy3-labelled cRNA was transcribed using the QuickAmp Labeling Kit (Agilent). According to the manufacturer's recommendations, One-Color RNA Spike-In Kit (Agilent) was used as spike-in control. 600 ng labelled cRNA were hybridized to custom 8x15k microarrays from Agilent (GEO platform accession GPL15859), which contained 2-4 probes for 6203 *C. albicans* genes, as well as 20 probes each for 10 spike-in controls and 336 probes for hybridsation and grid controls. The microarrays were scanned on a G2565A scanner (Agilent) and feature extraction and quality control were performed in Feature Extraction 10.7.3.1 (Agilent) using the protocol 'GE1_107_Sep09'. Microarray design and hybridzation, as well as image analysis were performed at the Microarray Core Facility of the HZI, Braunschweig.

Data from three biologically independent experiments were analyzed using the R/Bioconductor

-package limma (Gentleman *et* al., 2004; Smyth, 2004). Between-array-normalization by a quantile-method (Bolstad *et al.*, 2003; Smyth and Speed, 2003) was followed up by averaging the intensities of replicate probes and calculation of the logarithmic fold-change and an empirical Bayes moderated t-statistic (Smyth, 2004). Gene annotation data were from Candida Genome Database (Arnaud *et al.*, 2012; C_albicans_SC5314_version_A21-s02-m03-r02_chromosomal_feature.tab). The raw data and results can be accessed at GEO (accesion GSE39715).

Gene expression analysis by RT-PCR

For the working culture the pre-culture was diluted to OD_{620} = 0.2 in YNB supplemented with or without up to 20 µg ml^{-1} fludioxonil. After cultivation at 30 °C for 30 min, the cells were harvested, shock-frozen in liquid nitrogen and RNA was isolated, as described above.

3 µg of total RNA was employed in reverse transcription, with superscript II RT, random and oligo-dT_{12-18} primers, according to the manufacturer's recommendations (Invitrogen). Quantitative real-time PCR was carried out on a 96-well LightCycler® 480 system using the LightCycler® 480 SYBR Green I Master (Roche), as recommended by the manufacturer (95 °C, 60 °C, and 72 °C for 10 s each for 45 cycles). Gene sequences were obtained from the *Candida* Genome Database (Inglis *et al.*, 2011), and gene-specific oligonucleotides (Table S3) were designed by Roche's Probe Library Assay Design Center and synthesized by Eurofins MWG Operon. Specificity was controlled against the *C. albicans* genome sequence by using BLAST. Real-time analysis data (crossing-points) were normalized with respect to the actin gene *ACT1* and relative gene expression levels were calculated. The average and standard deviations of the gene expression levels relative to solvent controls in three independent experiments were calculated, and the significance of the changes in gene expression was tested by Student's t-test of normalized data ($p < 0.05$).

Determination of rhodamine 6G in supernatants

Uptake and efflux of rhodamine 6G (R6G) in *C. albicans* was determined according to a published protocol (Maesaki *et al.*, 1999). In short, *C. albicans* was precultured as described above. Cells from an exponentially growing culture were washed twice and suspended with an OD_{620nm} of 1 in 20 ml PBS. After starvation for 1 h at 30 °C, 10 µM R6G (equivalent to 4.8 µg ml^{-1}) was added and let diffuse and enrich in *C. albicans* for 30 min (diffusion phase). Cells from 12 ml suspension were then collected by centrifugation and resuspended in YNB to enable ATP production. Throughout the next 90 min (efflux phase), as well as the preceding diffusion phase, samples of 1 ml were taken, centrifuged for 1 min at 13,000 g and the supernatant was analyzed photometrically for

absorbance at 527 nm. A dilution series of R6G was used as a standard for the determination of the concentration.

Results and Discussion

Fludioxonil caused a mild inhibition of growth and germ tube formation in *C. albicans* SC5314

We had previously shown that the type III histidine kinase of *C. albicans*, Nik1p is the target of fludioxonil (Buschart *et al.*, 2012). However, inhibition of growth of *C. albicans* by fludioxonil is strongly dependent on the genetic background of the strain (Wesolowski *et al.*, 2010). Growth of isolate ATCC-10231 was severely inhibited by concentrations above 5 µg ml^{-1}, while growth of the isolate DSM-1577 and of SC5314, which is most commonly used in genetic studies, were only affected at high concentrations (Fig. 1 (a)).

It was reported that Nik1p is necessary for full yeast-to-hyphae transition (Alex *et al.*, 1998; Yamada-Okabe *et al.*, 1999). For this reason, morphological analyses of *C. albicans* treated with fludioxonil were conducted. When *C. albicans* SC5314 was grown under hyphae-inducing conditions, i. e. pH 7 and 37 °C, fludioxonil caused a slight reduction in germ tube formation and growth during the first four hours of induction (Fig. 1 (b) shows data from 2 h).

Fludioxonil induced the expression of *CDR1* and *CDR2*

To further characterise the effects of fludioxonil on *C. albicans*, changes in the gene expression of *C. albicans* grown under hyphae inducing conditions in response to treatment with 20 µg ml^{-1} fludioxonil were analyzed. The presence of fludioxonil lead to the differential expression of relatively few genes, with only 32 genes having positive Bayes posterior log odds-ratios of differential expression (column B in Tab. S1 and Tab. S2). Among the top 20 genes ranked by the significance of their differential expression (Tab. S1), only 10 genes were annotated with a putative or proven function. In accordance with the relevance of the fludioxonil-target Nik1p for germ tube formation, the gene for a known indicator of hyphal formation, hyphal wall protein 1 (*HWP1*; Staab *et al.*,1996; Staab and Sundstrom, 1998), was downregulated under fludioxonil treatment. We also observed the induction of ABC-transporter genes *CDR1* and *CDR2*. Below the threshold for significance, *CDR4* was also induced, while other important transporter genes, such as *MDR1* were not differentially expressed.

Due to the relevance of the ABC-transporter Cdr1p and Cdr2p for the resistance to antimycotics we

analyzed the expression of their genes under culture conditions favouring yeast growth in more detail. The expression of both *CDR1* and *CDR2* was dependent on the concentration of fludioxonil (Tab. 1). Concentrations as low as 2.2 µg ml^{-1} led to an up-regulation of *CDR1*, hence the active concentrations for this induction were lower than the concentrations that led to a significant growth inhibition.

However, the induction of the ABC-transporter genes was independent of the presence of the histidine kinase Nik1p – which is the target of fludioxonil – as induction was also observed in the deletion mutant Δ*nik1* (Fig. 2). Therefore, the induction of *CDR1* and *CDR2* is to be considered a target-independent side effect of fludioxonil in *C. albicans*.

CDR1 and *CDR2* were expressed at a basal level similar to the wild-type in a Δ*tac1* mutant (data not shown). Induction of *CDR2* expression by fludioxonil was completely dependent on the regulator Tac1p (Fig. 2), while *CDR1* was still induced significantly ($p < 0.05$) in the absence of Tac1p by fludioxonil, yet to a lesser extent than in the wild type. This is in agreement with the earlier finding that Tac1p is not essential for a basal Cdr1p-expression (Coste *et al.*, 2004) and that further factors can be involved in the inuction of *CDR1* (Shukla *et al.*, 2011).

In contrast to the herbicides acetochlor, metolachlor, dimethenamide and glyphosate, which were shown by Schmidt *et al.* (2008) to induce *CDR*-expression at concentrations also fungicidal or fungistatic, fludioxonil led to an induction of *CDR1* already at subinhibitory concentrations. These active concentrations were in the ranges of allowed (up to 20 µg ml^{-1}), as well as occasionally observed (up to 1 µg ml^{-1}), residual concentrations on fruit (Commission Regulation (EU) No 813/2011; Electronic Code of Federal Regulations, 40 C.F.R. § 180.516 (2012)).

Aggravation of fludioxonil-activity by deletion of *CDR1*

As both ABC-transporter genes *CDR1* and *CDR2* had been upregulated in response to fludioxonil, the effect of these transporters on the activity of fludioxonil was investigated using deletion mutants. Growth of SC5314 yeast cells was inhibited only slightly and transiently by fludioxonil, and similar results were found in deletion mutants of *CDR1* and *CDR2* (data not shown). We also tested the effect of fludioxonil on hyphae formation. As shown in Fig. 1 (b), fludioxonil strongly reduced the ability to form germ tubes in the Δ*cdr1* and Δ*cdr1,2*-mutants. The Δ*cdr2*-mutant was affected only in a similar degree as the wild type. This indicated that the fungicide was exported out of the cell by Cdr1p in wild type cells. While the induction of *CDR2*-expression by fludioxonil was stronger than that of *CDR1*, *CDR1* seemed to play a more important role in this process, as the Δ*cdr2*-mutant behaved similar to the wild type. This finding is similar to the case of fluconazole, where *CDR1* plays the bigger role in resistance (Tsao *et al.*, 2009).

Treatment of a mutant strain carrying a deletion in the gene *TAC1*, which codes for the known regulator of *CDR1*, also caused only a slight reduction in germ tube formation. This was in accordance with the partial induction of *CDR1*-expression in the Δ*tac1*-mutant and the basal expression of *CDR1* in this mutant.

Fludioxonil influenced the export rate of rhodamine 6G

The potential role of fludioxonil in drug resistance was evaluated by studying the effect of fludioxonil on the export efficiency of the ABC-transporters in extrusion of a known cargo, rhodamine 6G (R6G; Maesaki *et al.*, 1999).

4.8 µg ml^{-1} R6G was added to de-energized cells and taken up by passive diffusion, as observed via the absorbance of the culture-supernatant. The rapid diffusion rate was unchanged in the presence of 20 µg ml^{-1} fludioxonil in comparison to control cultures as the supernatant contained less than 250 ng ml^{-1} R6G in each case after 30 min. It was also the same for the wild type and the Δ*nik1* mutant.

After transfer into glucose-containing medium, *C. albicans* was able to produce ATP needed for the export of R6G by the ABC-transporters. Consequently, R6G was exported actively with an extrusion rate higher than the inward diffusion, resulting in a net efflux, which was visible from the increase in absorbance of the supernatant. Cultures, which were treated with fludioxonil during the efflux phase, displayed a decreased R6G efflux rate in comparison to control cultures (Fig. 3 (a)). Fludioxonil, which had been added during the diffusion phase of the experiment, had the same effect as fludioxonil added during the efflux-phase of the experiment (data not shown). However, R6G efflux was increased, if the pre-cultures had been treated with fludioxonil before loading with R6G (Fig. 3 (a)).

These results can be explained by competition of R6G with fludioxonil for binding sites within the ABC-transportes. When fludioxonil and R6G are added simultaneously to *C. albicans*, only the basal efflux capacity is available for both compounds. This resulted in a decelerated efflux rate for R6G. Pre-incubation of the cells with fludioxonil before starvation and loading with R6G led to the increased expression of *CDR1*, and thus, increased efflux capacity. This could be observed after supplying the cells with glucose as an energy source.

The changes in R6G-efflux rates in the presence of fludioxonil observed in the wild type were also detected in the Δ*nik1* mutant (Fig. 3 (b)), pointing again to the fact that this effect is independent of Nik1p.

The growth inhibitory effect of rhodamine 6G was not significantly altered by fludioxonil

As R6G is toxic to *C. albicans* and could not be extruded as effectively by *C. albicans* simultaneously treated with fludioxonil, the effect of fludioxonil on the toxicity of R6G was examined. Only with high concentrations of fludioxonil, a slight increase of the growth inhibitory effect of R6G was detected (Tab. 1). Therefore, the attenuation of R6G export by fludioxonil did not lead to a synergistic effect between the two substances.

The resistance to fluconazole was increased by fludioxonil

Another known cargo of the ABC-transporters of *C. albicans* is the antimycotic fluconazole (Sanglard *et al.*, 1995). As the ABC-transporters play a vital role in resistance development against this drug, the effect of fludioxonil on the susceptibility to fluconazole was also analyzed. Concurrent exposition of *C. albicans* SC5314, as well as the fludioxonil resistant isolate DSM-1577, to fludioxonil and fluconazole led to an increase in fluconazole resistance by approximately a factor of 3 (Tab. 1). This effect was also observed in a standardized susceptibility assay using the EUCAST method (EUCAST, 2008), where a change of MICs from 0.5 µg ml^{-1} (control) to 4 µg ml^{-1} (treated with 20 µg ml^{-1}) was observed. The effect was dependent on the concentration of fludioxonil and the active concentration range corresponded to the concentration range that was sufficient to trigger induction of *CDR1* expression. Thus, like the induction of *CDR1* expression, increased resistance to fluconazole was caused by fludioxonil concentrations within allowed maximum residue levels.

While the exposure to fludioxonil led to a decrease in susceptibility to fluconazole, the activity of rhodamine 6G was slightly enhanced, as described above. This may be explained by an interaction of fluconazole and R6G with different subdomains of the ABC-transporters, which was revealed by a recent study of hybrid ABC-transporters (Tanabe *et al.*, 2011). Therefore, fludioxonil may compete with R6G for binding sites and the binding site for fludioxonil seems to be closer related to that of R6G.

The fludioxonil-induced decrease in fluconazole susceptibility was independent of Nik1p, but *CDR1* was essential for fluconazole resistance

The factors responsible for the increase of resistance to fluconazole due to fludioxonil exposure were further analyzed using deletion mutants. An earlier study had indicated the histidine kinase Chk1p and a response regulator, Ssk1p, from *C. albicans* to be involved in resistance to fluconazole, as the uptake of the drug and thus the intracellular concentrations of the drug, as well as the susceptibility were increased in knock-out mutants of the respective mutants (Chauhan *et al*., 2007). However, the basal susceptibility to fluconazole was alike in the wild type and *Δnik1*-

mutant. Moreover, we observed a fludioxonil-triggered increase of fluconazole resistance, which was in accordance with the induction of *CDR1* and *CDR2*. *W*e assume this increased the efflux capacity for fluconazole. Both observations were independent of the presence of the histidine kinase Nik1p, which is the known target of fludioxonil (Tab. 2). The difference of the roles of Nik1p and Chk1p in fluconazole susceptibility is likely due to activation of different signal transduction pathways, which is generally assumed because of phenotypical differences in the respective deletion mutants (Chauhan and Calderone, 2008).

The antagonistic effect of fludioxonil was observed in all analyzed deletion mutants with the exception of those carrying deletions in *CDR1* (Tab. 2). The Δ*cdr1* mutants were, as expected, hypersensitive to fluconazole. The decrease in susceptibility to fluconazole caused by fludioxonil was not dependent on the known activator of *CDR1* expression Tac1p. This was in agreement with the finding that *CDR1* was expressed and at least partially induced by fludioxonil in a Δ*tac1* mutant (Fig. 2). As the involvement of further transcription factors in *CDR1*-induction has been shown (Shukla *et al.*, 2011), fludioxonil-triggered *CDR1*-induction is likely promoted by another transcription factor in addition to Tac1p.

Conclusion

Exposure of *C. albicans* to the fungicide fludioxonil triggered a concentration dependent induction of the expression of ABC-transporter genes *CDR1* and *CDR2*, which are involved in resistance to antimycotics such as fluconazole. As a consequence the resistance of *C. albicans to* fluconazole increased, so that fludioxonil indirectly antagonized the activity of fluconazole. This induction effect occurred at sub-growth-inhibitory concentrations of the fungicide. It was also independent of the primary target of fludioxonil in *C. albicans*, the histidine kinase Nik1p, which is involved in morphological changes as well as in stress response.

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Tables

Table 1: Concentration dependent effects of fludioxonil on growth, the expression of *CDR1* and *CDR2* and the susceptibility to fluconazole and rhodamine 6G.

	DSM-1577	SC5314			
Fludioxonil [µg ml^{-1}]	IC_{50}† ± sd [µg ml^{-1}]	IC_{50}† ± sd [µg ml^{-1}]		Regulation‡ [log_2 fold change ± sd]	
	fluconazole	fluconazole	rhodamine 6G	*CDR1*	*CDR2*
0	0.1 ± 0.0	0.3 ± 0.1	0.5 ± 0.2	-§	-§
0.25	0.2 ± 0.2	0.5 ± 0.3	0.6 ± 0.1	1.0 ± 0.5	0.4 ± 0.4
0.74	0.3 ± 0.3	0.7 ± 0.1*	0.6 ± 0.1	2.2 ± 1.9	1.6 ± 1.4
2.2	0.5 ± 0.4	0.7 ± 0.1*	0.5 ± 0.1	2.3 ± 0.8*	4.5 ± 2.2
6.7	0.5 ± 0.3*	0.7 ± 0.2*	0.5 ± 0.2	3.0 ± 1.1*	6.4 ± 2.0*
20	0.6 ± 0.1*	0.9 ± 0.1*	0.5 ± 0.2	3.2 ± 0.8*	5.5 ± 1.7*

†IC_{50} values were determined photometrically after growth of 5000 cells per ml for 24 h.

‡ Gene expression as compared to untreated cells was measured by qRT-PCR after 30 min treatment.

§ Reference samples for gene expression.

* $p < 0.05$

sd: standard deviation

Table 2:

Effect of fludioxonil on the susceptibility of *C. albicans* deletion mutants to fluconazole.

Fludioxonil [µg ml^{-1}]	IC_{50} ± sd of fluconazole† [µg ml^{-1}]					
	SC5314	Δ*nik1*	Δ*cdr1*	Δ*cdr2*	Δ*cdr1*,Δ*cdr2*	Δ*tac1*
0	0.3 ± 0.1	0.3 ± 0.0	<0.05	0.2 ± 0.1	0.08 ± 0.01	0.4 ± 0.2
0.25	0.5 ± 0.3	0.6 ± 0.2	<0.05	0.6 ± 0.1*	0.08 ± 0.01	0.8 ± 0.2*
0.74	0.7 ± 0.1*	0.7 ± 0.3	<0.05	0.7 ± 0.0*	0.08 ± 0.01	0.9 ± 0.1*
2.2	0.7 ± 0.1*	0.7 ± 0.1*	<0.05	0.8 ± 0.1*	0.08 ± 0.01	0.7 ± 0.2
6.7	0.7 ± 0.2*	0.7 ± 0.4	0.05 ± 0.02	0.9 ± 0.1*	0.09 ± 0.01	0.8 ± 0.1*
20	0.9 ± 0.1*	0.9 ± 0.4	0.08 ± 0.01	1.2 ± 0.7	0.09 ± 0.02	0.7 ± 0.1

† IC_{50} values were determined photometrically after growth of 5000 cells per ml for 24 h.

*$p < 0.05$

sd: standard deviation

Figure legends

Figure 1: (a) Growth inhibitory effect of fludioxonil is dependent on the strain of *C. albicans*. Wild type isolates SC5314 (circles), ATCC-10231 (squares) and DSM-1577 (diamonds) were incubated with a dilution series of fludioxonil for 24 hours, before growth (displayed as % of untreated control) was determined photometrically. (b) Effect of 20 µg ml^{-1} fludioxonil (grey bars) on the germ tube formation of *C. albicans* SC5314 and deletion mutants; white bars; untreated controls. Data are from microscopical analysis after 2 h of incubation in RPMI-1640 at 37 °C. Fludioxonil caused a strong inhibition of germ tube formation in strains lacking *CDR1*. Data of 750 to 2200 cells from two independent experiments are shown. Total cell numbers are set as 100 %.

Figure 2: Induction of *CDR1* and *CDR2* expression in response to treatment with 20 µg ml^{-1} fludioxonil in *C. albicans* SC5314 (WT) and deletion mutants Δ*nik1* and Δ*tac1*. Fludioxonil-dependent induction of *CDR1* and *CDR2* was independent of Nik1p. Data from three independent experiments.

Figure 3: Effect of fludioxonil on the active Cdr-mediated export of rhodamine 6G. R6G was photometrically detected in the supernatant as described in the materials and methods section. (a) Treatment with fludioxonil during the efflux phase (triangles) decreased the efflux rate in comparison to control cultures (filled circles), while treatment of the preculture with fludioxonil increased Cdr-activity (empty circles); data from one representative of at least three experiments are shown. (b) The decrease of Cdr-efficiency in the presence of fludioxonil observed in the wild type (white bars) was also detected in the Δ*nik1* mutant (grey bars); data after 60 min efflux from three independent experiments.

Figures

Figure 1:

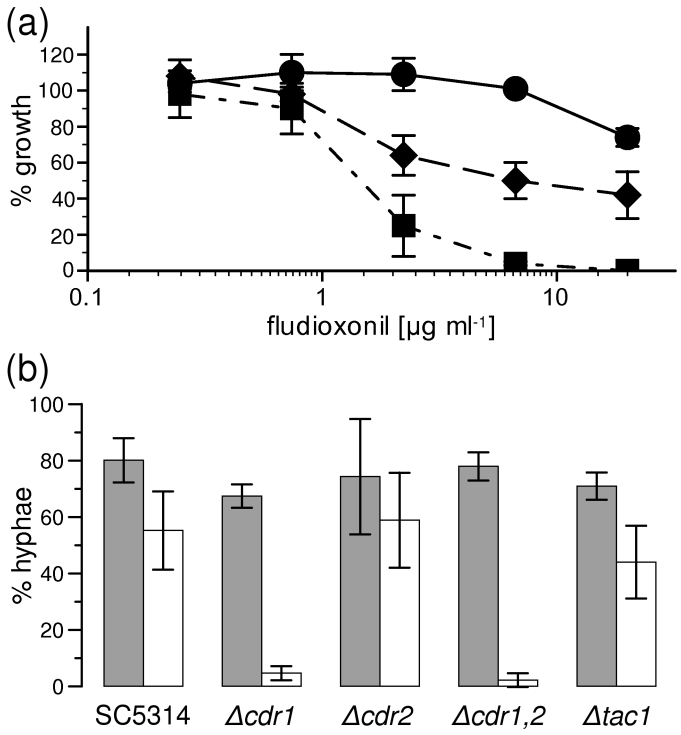


Figure 2:

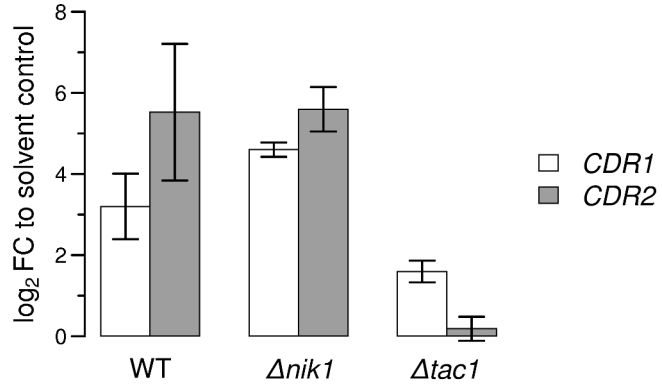
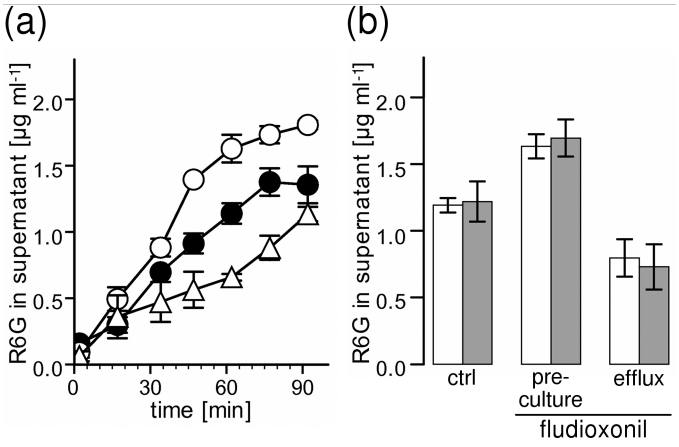


Figure 3:


(a)
(b)
R6G in supernatant [µg ml-1]
2.0
1.5
1.0
0.5
0.0
0
30
60
90
time [min]
ctrl
pre-culture
efflux
fludioxonil